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(54) Title: LEUKOLYSIN/MMP25/MT6-MMP

(57) Abstract: A novel compound, leukolysin, and therapeutic methods for treating conditions associated with the presence or absence of leukolysin is provided. Also provided are methods to detect or monitor inflammatory disease by determining the presence or amount of leukolysin in a physiological sample.

LEUKOLYSIN/MMP25/MT6-MMP

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Abbreviations

The following abbreviations are used throughout the present document: MMP: matrix metalloproteinase; ECM, extracellular matrix; MT: membrane-type; PC, proprotein convertase; PMN, polymorphonuclear leukocytes; GAPDH, glyceradehyde-3-phosphate dehydrogenase; IL: interleukin; GPI-PLC, glycosyl-phosphatidylinositol phospholipase C; GFP, green fluorescent protein.

Background of the Invention

Neutrophils (PMNs) are the first cells to arrive at the site of infection and play a critical role in host defense against invading microbes (Weiss 1989). In the process of resolving ongoing infection, they deploy a powerful arsenal of molecules to destroy the infectious agent as well as the infected cells and tissues, thus, resulting in host tissue damage (Weiss 1989, Birkedal-Hansen 1993). Among proteolytic enzymes identified in the PMNs, the matrix metalloproteinases (MMPs) have attracted considerable attention for their proposed role in the destruction of extracellular matrix under physiological and pathological conditions (Weiss 1989, Birkedal-Hansen 1993).

The first MMP identified in PMNs was MMP-8 or neutrophil collagenase that is stored in specific granules and is capable of cleaving type I collagen into typical ¼ and ¾ fragments (Lazarus 1968, Murphy 1977, Tschesche 1995, Hasty 1990). MMP-9, originally detected in an alveolar macrophage, is also known as the gelatinolytic activity discharged by PMNs (Mainardi 1984, Hibbs 1985, Wilhelm 1989). Although expressed at relatively high levels in PMNs, both MMPs are no longer considered PMN-specific due to their widespread

expression by cells from other lineages (Tschesche 1995, Mainardi 1984, Schonermark 1996, Shlopov 1997, Hanemaaijer 1997). Furthermore, MMP-9 deficient mice appear to produce fully functional neutrophils, but exhibit deficit in angiogenesis during endochondral ossification (Vu 1998, Betsuyaku 1990). Liu and colleagues, however, demonstrated that MMP-9 deficient mice are resistant to blister formation due to deficiency in neutrophil-mediated inactivation of α1-proteinase inhibitor (α1PI) in a murine bullous hemphigoid model (Liu 2000).

Members of the MMP superfamily have been consistently implicated in the destruction of ECM by virtue of their ability to destroy all ECM components under physiologically relevant conditions (Werb 1998, Woessner 1991, Weiss 1989, Massova 1998). Evolved from modular domains and motifs through exon-shuffling, each MMP possesses common conserved structural domains such as those for catalysis and unique motifs like the transmembrane domains. Although all MMPs contain signal peptides for secretion, the defining feature for these enzymes is a catalytic domain of 170 or so amino acids centered around a zinc-binding motif HEXXHXXXXXH followed by a Met turn (Stocker 1995, Nagase 1999). To keep these powerful proteinases in latent form, most MMPs rely on a conserved cysteine residue in the prodomains to interact with the catalytic zinc according to the cysteine-switch latency model (Van Wart 1990). The only exception is the newly identified CA-MMP which lacks a cysteine-switch in the prodomain (Pei 1999a). At the carboxyl-termini, a hemopexin-like domain is found in all MMPs except CA-MMP and matrilysin (Pei 1999a, Muller 1988, Velasco 1999). Recently, two additional motifs have been recognized within the MMP family: 1) a furin- or proprotein convertase- recognition motif, RXKR/R for zymogen activation (Pei 1995, Santavicca 1996, Steiner 1998); and 2) a hydrophobic segment at the carboxyl termini of MMP14-17 consistent with that of a transmembrane domain (Sato 1994, Takino 1995, Will 1995, Puente 1996). Based on these functional domains, a diverse array of MMPs have evolved to accomplish the complex ECM remodelings associated with the growth and development of multicellular organisms such as human.

There are several neutrophil-associated pulmonary diseases, including chronic obstructive pulmonary disease (COPD), chronic bronchitis (CB), pulmonary emphysema, α-1 anti-trypsin deficiency, cystic fibrosis, idiopathic pulmonary fibrosis, and adult respiratory distress syndrome.

COPD is a complex condition with an imprecise definition, which makes a definitive morphological description difficult. Three conditions contribute to COPD. In chronic bronchitis there is cough and mucous hypersecretion with enlargement of tracheobronchial submucosal glands and a disproportionate increase of mucous acini. CD8+ve lymphocytes predominate over CD4+ve cells and there are increased numbers of subepithelial macrophages and intra-epithelial neutrophils. Exacerbations of bronchitis are associated with a tissue eosinophilia, apparent absence of IL-5 protein but gene expression for IL-4 and IL-5 is present. In small or peripheral airways disease, there is inflammation of bronchioli and mucous metaplasia and hyperplasia, with increased intraluminal mucus, increased wall muscle, fibrosis, and airway stenoses (also referred to as chronic obstructive bronchiolitis). Respiratory bronchiolitis involving increased numbers of pigmented macrophages is a critically important early lesion. Increasingly severe peribronchiolitis includes infiltration of T lymphocytes in which the CD8+ subset again predominates. These inflammatory changes may predispose to the development of centrilobular emphysema and reduced FEV1 via the destruction of alveolar attachments. In emphysema there is abnormal, permanent enlargement of airspaces distal to the terminal bronchiolus (*i.e.* within the acinus) accompanied by destruction of alveolar walls and without obvious fibrosis. The severity of emphysema, rather than type, appears to be the most important determinant of chronic deterioration of airflow, and in this there may be significant loss of elastic recoil and microscopic emphysema prior to the observed macroscopic destruction of the acinus. Airway obstruction and chronic expectoration, as well as accelerated decline in lung function, are associated with increased numbers of neutrophils in the sputum of smokers and ex-smokers. This suggests that neutrophilic inflammation of the airways may be involved in the pathogenesis of COPD.

The role of glucocorticoids in the treatment of chronic obstructive pulmonary disease (COPD) is controversial. Studies have shown that inhaled steroids have little anti-inflammatory effect, and that the inflammatory process in COPD is resistant to the antiinflammatory effect on glucocorticoids. (Barnes 1999; Keatings 1997; Culpitt 1999). At this time, there is no effective anti-inflammatory therapy for these patients.

Pulmonary emphysema is defined as an abnormal, permanent enlargement of the air spaces distal to the terminal bronchioles with destruction of the air space walls, but without obvious fibrosis. Central to the pathogenesis of emphysema is lung destruction resulting from inadequate protection of the alveoli against enzymes released by inflammatory cells. The proteolytic enzymes implicated in the development of emphysema include neutrophil elastase, a major serine protease contained within neutrophil granules.

In α_1-Antitrypsin Deficiency disease, there is a deficiency of the enzyme neutralizing neutrophil elastase, namely α_1-antitrypsin. Moreover, there is evidence that the numbers of neutrophils migrating into the lung parenchyma is increased. Because of the inadequate defenses against neutrophil elastase, uninhibited neutrophil elastase is free to interact with substrates and inflammatory cells activating them. The result of this process is proteolytic destruction by neutrophil elastase of the fragile alveolar walls culminating in a gradual destruction of alveoli. This process is strikingly accelerated in cigarette smokers.

Cystic fibrosis is a common hereditary disorder of Caucasians and represents the most aggressive form of bronchitis known. Respiratory manifestations of cystic fibrosis develop at an early age even in the first year of life. Frequent respiratory infections occur with production of thick, sticky sputum. The clinical course is punctuated by acute exacerbations of inflammation and infection of the airways with progressive deterioration of airway function. The pulmonary inflammation typical of cystic fibrosis resembles that of α_1-antitrypsin deficiency and the epithelial surface of the lung in both disorders is burdened by neutrophil elastase and oxidants. However, in cystic fibrosis the bronchial inflammation is much more intense and the numbers

of neutrophils in the airway epithelial lining fluid may be 500 times greater than in normal individuals. Although the pathogenesis of airway inflammation in patients with cystic fibrosis remains the subject of debate, nonetheless neutrophil elastase is implicated as playing a critical role. In cystic fibrosis, the normal protective defenses against proteases are intact, but the inflammation on the airway epithelial surface is so intense that these defenses against neutrophil elastase are overwhelmed and rendered ineffective.

The bronchitis associated with cystic fibrosis is the most aggressive form of bronchitis known, but the bronchitis associated with cigarette smoking is by far the most common. The population of inflammatory cells includes increased numbers of neutrophils, and they release an increased burden of oxidants and proteases including neutrophil elastase on the airway epithelial surface. Here, as in cystic fibrosis, it is assumed that the defenses of the airway against proteases are overwhelmed by the inflammation, albeit not to the extent as in cystic fibrosis.

The etiology of idiopathic pulmonary fibrosis is unknown, however, evidence exists that these patients show an inflamed lower respiratory tract even before the development of frank fibrosis. Among the inflammatory cells, neutrophils are prominent. It is believed that activated inflammatory cells damage alveolar structures by releasing oxidants and proteases, and that these reactants play a major role in injury to the epithelium and endothelium in idiopathic pulmonary fibrosis.

Considerable evidence links inflammation to the pathology of Acute (Adult) Respiratory Distress Syndrome (ARDS). Moreover, the neutrophil, in particular, with its extensive armamentarium of toxins, which can injure and destroy host tissue, has been implicated as an important mediator of ARDS.

Therefore, there is a driving need to develop new and effective treatments and compounds for treating inflammatory conditions, such as arthritis and asthma, neutrophil-associated pulmonary diseases such as COPD, CB, pulmonary emphysema, α-1 anti-trypsin deficiency, cystic fibrosis, idiopathic pulmonary fibrosis, and adult respiratory distress syndrome. There is also a need for effective treatments and compounds for stimulating the activation of

neutrophils and/or for mediating the destruction of invading pathogens and infected host tissues. There is also a need for compounds that inhibit the activation of neutrophils and/or the over-reaction of the immune system. There is further a need for methods for administering such compounds in animals.

Summary of the Invention

The present invention provides a polypeptide that is matrix metalloproteinase 25 (MMP25, also called MT6-MMP or leukolysin). The term "MMP25" includes variants or biologically active or inactive fragments of MMP25, including one or more of the following domains of MMP25: the pro-, catalytic, or pexin domain of MMP25, or the GPI anchor region of MMP25. A "variant" of MMP25 is a polypeptide or oligopeptide MMP25 that is not completely identical to native MMP25. Such a variant MMP25 can be obtained by altering the amino acid sequence by insertion, deletion or substitution of one or more amino acid. The amino acid sequence of the protein is modified, for example by substitution, to create a polypeptide having substantially the same or improved qualities as compared to the native polypeptide. The substitution may be a conserved substitution. A "conserved substitution" is a substitution of an amino acid with another amino acid having a similar side chain. A conserved substitution would be a substitution with an amino acid that makes the smallest change possible in the charge of the amino acid or size of the side chain of the amino acid (alternatively, in the size, charge or kind of chemical group within the side chain) such that the overall peptide retains its spacial conformation but has altered biological activity. For example, common conserved changes might be Asp to Glu, Asn or Gln; His to Lys, Arg or Phe; Asn to Gln, Asp or Glu and Ser to Cys, Thr or Gly. Alanine is commonly used to substitute for other amino acids. The 20 essential amino acids can be grouped as follows: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine having nonpolar side chains; glycine, serine, threonine, cystine, tyrosine, asparagine and glutamine having uncharged polar side chains; aspartate and glutamate having acidic side chains; and lysine, arginine, and histidine having basic side chains. Stryer (1981) p. 14-15; Lehninger (1975) p. 73-75.

The amino acid changes are achieved by changing the codons of the corresponding nucleic acid sequence. It is known that such polypeptides can be obtained based on substituting certain amino acids for other amino acids in the polypeptide structure in order to modify or improve biological activity. For example, through substitution of alternative amino acids, small conformational changes may be conferred upon a polypeptide which result in increased activity or enhanced immune response. Alternatively, amino acid substitutions in certain polypeptides may be used to provide residues which may then be linked to other molecules to provide peptide-molecule conjugates which retain sufficient properties of the starting polypeptide to be useful for other purposes.

One can use the hydropathic index of amino acids in conferring interactive biological function on a polypeptide, wherein it is found that certain amino acids may be substituted for other amino acids having similar hydropathic indices and still retain a similar biological activity. Alternatively, substitution of like amino acids may be made on the basis of hydrophilicity, particularly where the biological function desired in the polypeptide to be generated in intended for use in immunological embodiments. The greatest local average hydrophilicity of a "protein", as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity. U.S. Patent 4,554,101. Accordingly, it is noted that substitutions can be made based on the hydrophilicity assigned to each amino acid.

In using either the hydrophilicity index or hydropathic index, which assigns values to each amino acid, it is preferred to conduct substitutions of amino acids where these values are ±2, with ±1 being particularly preferred, and those with in ±0.5 being the most preferred substitutions.

The variant MMP25 has at least 50%, preferably at least about 80%, and more preferably at least about 90% but less than 100%, contiguous amino acid sequence homology or identity to the amino acid sequence of a corresponding native MMP25.

The amino acid sequence of the variant MMP25 polypeptide corresponds essentially to the native MMP25 amino acid sequence. As used herein "correspond essentially to" refers to a polypeptide sequence that will elicit a

biological response substantially the same as the response generated by native MMP25. Such a response may be at least 60% of the level generated by native MMP25, and may even be at least 80% of the level generated by native MMP25. An immunological response to a composition is the development in the host of a cellular response to the polypeptide of interest.

A variant of the invention may include amino acid residues not present in the corresponding native MMP25 or deletions relative to the corresponding native MMP25. A variant may also be a truncated "fragment" as compared to the corresponding native MMP25, *i.e.*, only a portion of a full-length protein. For example, the variant MMP25 may vary from native MMP25 in that it does not contain a cell wall insert. MMP25 variants also include peptides having at least one D-amino acid.

The variant MMP25 of the present invention may be expressed from an isolated DNA sequence encoding the variant MMP25. For example, the variant MMP25 may vary from native MMP25 in that it does not contain a signal sequence or a cell wall insert. "Recombinant" is defined as a peptide or nucleic acid produced by the processes of genetic engineering. The terms "protein," "peptide" and "polypeptide" are used interchangeably herein.

The present invention also provides a polynucleotide sequence encoding, or complementary to, MMP25. It further provides an expression vector containing a polynucleotide encoding MMP25, and a transgenic cell containing an expression vector containing a polynucleotide encoding MMP25.

The present invention also provides a method for treating a leukolysin-associated condition by administering to an afflicted animal, particularly a human, an amount of a therapeutic preparation containing a compound effective to counteract the symptoms of the condition. The inhibitory compound may be TIMP-1, TIMP-2, TIMP-3, TIMP-4 or their closely related inhibitors; a BB-94 inhibitor; catalytically inactive leukolysin; hemopexin-like domain of leukolysin; or natural herb compounds that bind zinc, thereby removing zinc from leukolysin. These compounds may be isolated from naturally-occurring sources, or may be synthetically produced. Further, the compound may be full-length as compared to the naturally-occurring form, or may be modified in some manner,

provided they have similar biological activity. The leukolysin-associated condition may be 1) acute or chronic inflammation caused by neutrophils; 2) autoimmune diseases where many neutrophil proteins are autoantigens; 3) asthma other related immune disorders caused by granulocytes; 4) tissue injury and organ failure mediated by MMP25 expressing cells such as granulocytes; 5) malignant cancers where MMP25 enables tumor cells to invade and metastasize through its ability to degrade extracellular matrix; 6) genetic disorders with defects in neutrophil deficiency; 7) organ transplant patients with episodes of granulocyte infiltrations; 8) organ failure (such as renal failure) due to attacks by neutrophils or neutrophil-related immune responses; 9) cardiovascular conditions or damages mediated by neutrophils or its discharged contents; 10) lung damage mediated by neutrophils, such as with chronic obstructive pulmonary disease; 11) skin irritations and blister where granulocytes mediate the destruction of connected tissues; 12) allergy associated with eosinophils and mast cells; 13) arthritis; 14) chronic bronchitis; 15) pulmonary emphysema; 16) α-1 anti-trypsin deficiency; 17) cystic fibrosis; 18) idiopathic pulmonary fibrosis; 19) adult respiratory distress syndrome; 20) paroxysmal nocturnal hemoglobinuria, a disorder with GPI-anchor deficiency; 21) familial Mediterranean fever (a hereditary disease with recurrent inflammation); or 22) periodontitis and oral ulcers. The therapeutic preparation may be administered in combination with a pharmaceutically acceptable liquid vehicle. It may be administered by spraying or by nebulization.

The present invention also provides a method for stimulating the activation of neutrophils in an animal in need thereof, by administering to an animal a therapeutic amount of a composition containing a polypeptide having a sequence encoding MMP25 to the animal effective to stimulate the activation of neutrophils in the animal. The compound may be a cytokine or chemokine; a modified version of leukolysin exhibiting heightened activities; G-CSF or GM-CSF, which can upregulate leukolysin expression; or transcriptional factor in the class of C/EBPs, which controls the transcription of myeloid lineage. Further, the compound may be IL1, 2, 3, 4, 6, 7, 8, 9, 10, 11, or 12.

The present invention also provides a method for mediating the destruction of a pathogen or infected host tissues in an animal in need thereof, by administering to an animal a therapeutic amount of a composition having a polypeptide containing a sequence encoding MMP25 to the animal effective to mediate the destruction of the invading pathogens or the infected host tissues in the animal. The pathogen may be a virus, bacterium, fungus or parasite.

The present invention also provides a method for inhibiting the activation of neutrophils in an animal in need thereof, by administering to an animal a therapeutic amount of a composition having a polypeptide containing a sequence encoding MMP25 to the animal effective to inhibit the activation of neutrophils in the animal. The animal may have an inflammatory disease such as fever, periodontal disease, cystic fibrosis, familial Mediterranean fever, arthritis, or emphysema.

The present invention also provides a method for inhibiting an over-reactive immune system response in an animal in need thereof, by administering to an animal a therapeutic amount of a composition having a polypeptide containing a sequence encoding MMP25 to the animal effective to inhibit the over-reactive immune system response in the animal. The over-reactive immune system response may be caused by allergy or an autoimmune disease, such as arthritis.

The present invention provides a method of suppressing tumor invasion and metastasis by administering to individuals with malignancy a composition containing natural or synthetic inhibitors of MMP25, or an antibody specific for MMP25, or a poly- or oligo-nucleotide that can block the activity of MMP25, in order to attenuate the mobility of cells expressing MMP25.

The present invention provides a method of stimulating neutrophils by administering to an animal experiencing heavy infections a composition having a sequence encoding MMP25 to the animal effective to mediate the destruction of invading pathogens.

The present invention also provides an antibody that specifically binds isolated, purified MMP25. The antibody may be a monoclonal or polyclonal antibody. The fragment may be an Fab, $F(ab')_2$, or Fv fragment. The antibody

or fragment thereof may be attached to a substrate, such as a gel, hydrogel, resin, bead, nitrocellulose, nylon filter, microtiter plate, culture flask, or polymeric material. The antibody or fragment thereof may further have a detectable moiety, such as a radionuclide, enzyme, specific binding pair component, colloidal dye substance, fluorochrome, chemiluminescent substance, electrochemiluminescent substance, electroactive agent, reducing substance, latex, digoxigenin, metal, particulate, dansyl lysine, antibody, protein A, protein G, electron dense material, or chromophore.

The present invention further provides a pharmaceutical composition containing an amount of an antibody, a fragment thereof or a mixture thereof that specifically binds to MMP25, wherein the amount is effective to inhibit a neutrophil response in a recipient with acute or chronic inflammation and tissue injury.

The present invention provides a continuous cell line that produces an antibody (monoclonal or polyclonal) that specifically recognizes a target peptide, wherein the target peptide is leukolysin. The target peptide may be covalently linked to a carrier molecule, such as keyhole limpet hemocyanin (KLH).

The present invention provides an animal that produces polyclonal antibodies that specifically recognizes a target peptide, wherein the target peptide is leukolysin. The target peptide may be covalently linked to a carrier molecule.

The present invention provides a diagnostic method for detecting a leukolysin-associated condition in a patient having the steps of first contacting a physiological sample suspected of containing leukolysin to form a purified leukolysin sample with an amount of detection agent specific for leukolysin to form an leukolysin:detection agent complex; wherein the detection agent is an antibody or fragment thereof that specifically recognizes leukolysin; and then detecting the presence or amount of leukolysin:detection agent complex present in the sample to determine whether the patient has inflammatory disease. The physiological sample may be a plasma, serum, tears, urine, whole gut lavage, lung lavage, peritoneal lavage, skin blister fluids, mucus, feces, GI tract tissue including mucosa and submucosa, or jejunal effluent. The detection step may be by enzyme-linked immunosorbent assay, immunonephelometry, agglutination,

precipitation, immunodiffusion, immunoelectrophoresis, immunofluorescence, electrochemiluminescence, surface plasmon resonance, chemiluminescence, electrochemical immunoassay, radioimmunoassay, or immunohistochemistry.

The present invention provides a method for monitoring the treatment of leukolysin-related disease in a patient having the steps of first contacting a physiological sample suspected of containing leukolysin with an amount of detection agent specific for leukolysin to form an leukolysin:detection agent complex; second, detecting the amount of leukolysin:detection agent complex present in the sample; third, repeating the first two steps at a point later in time; and last, comparing the amounts determined in the second and third steps, and correlating the change in the amounts to determine whether inflammation is diminishing.

The present invention provides kit for the detection of inflammatory disease in a patient, where the kit contains a composition comprising a detection agent specific for leukolysin, wherein the detection agent is an antibody or fragment thereof that specifically recognizes the leukolysin peptide; and packaging materials enclosing the detection agent.

The term "treatment" as used herein includes any treatment of a condition or disease in a human, and includes inhibiting the disease or condition, (*i.e.* arresting its development), relieving the disease or condition (*i.e.* causing regression of the condition), or relieving the conditions caused by the disease (*i.e.* symptoms of the disease).

The term "therapeutically effective amount" refers to that amount which is sufficient to effect treatment, as defined herein, when administered to a human in need of such treatment. The therapeutically effective amount will vary depending on the subject and disease state being treated, the severity of the affliction and the manner of administration, and may be determined routinely by one of ordinary skill in the art.

Brief Description of the Figures

Figure 1: Leukolysin: Primary amino acid sequence, domain structure and alignments with MT-MMPs. Figure 1A: The amino acid sequence is shown with markings for the signal peptide cleavage site (downward arrow), putative zymogen activation cleavage site by furin (bold and italicized letters with a downward arrowhead), the conserved catalytic zinc binding domain (bold and underlined), the hemopexin-like domain bound by a pair of cysteines (bold and italicized), and the hydrophobic segment at the C-terminus (bold and underlined).

Figure 1B: A schematic representation of domain structure for leukolysin. The numbers on the top indicate the positions of the amino acid residues bordering various domains. S: signal peptide; Pro: prodomain; F: furin recognition motif; CAT: catalytic; H: hinge; Pexin: hemopexin-like.

Figure 1C: Sequence alignment between leukolysin (shown as MMP25) and the MT-MMP subgroup. The names of each gene are shown on the left column. The cysteine-switches and the catalytic domains are shown in bold letters. The furin cleavage sites are boxed and indicated by a downward arrow. The end of the catalytic domains and the beginning and end of the hemopexin domains are marked by vertical lines. The transmembrane domains are boxed. * indicates identity, : for strong homology and . for weak homology.

Figure 2: Leukolysin is expressed specifically by neutrophils. Figure 2A: Reverse transcribed cDNA (0.5 ng aliquot each) from human multiple tissue panels (Clontech, CA) including 26 different tissues and cell types as indicated at the top (lanes 1-26) were amplified to give rise to ~ 600 bp fragment of leukolysin catalytic domain. To control for the amount of cDNA in each reaction, the same panels are amplified for the housekeeping gene GAPDH and presented at the lower panel.

Figure 2B: Populations of lymphocytes (lane 3), neutrophils (lane 4) and monocytes (lane 5) (>97% pure) were separated from the peripheral blood leukocyte pool and total RNA (5 μg) were reverse transcribed (RT). An aliquot (2 μl from a total of 20 μl RT reaction) were subsequently amplified for GAPDH

(lower panel) and leukolysin (MMP25, upper panel) as described above. The control contains all reaction mixture except cDNA.

Figure 2C: Leukolysin is expressed as a 4.0 kb mRNA. Total RNAs isolated from neutrophils were analyzed by Northern blotting at 5 μg (lane 1), 10 μg (lane 2) and 20 μg (lane 3) per lane. A full-length leukolysin cDNA was radiolabeled as the probe for hybridization. The arrowhead on the right indicated the position for leukolysin (MMP25). The migration positions for ribosome 28 and 18s RNA are indicated on the left.

Figure 3: Stable expression of leukolysin$_{1-280}$. Figure 3A: Schematic diagram for the leukolysin expression construct. The upper portion indicates the full length leukolysin (MMP25) and the lower portion for leukolysin$_{1-280}$(MMP25cat) as a in-frame fusion with the FLAG tag as described in Materials and Methods for Example 1. S: signal peptide; Pro: prodomain; F: furin; CAT: catalytic; H: hinge; Pexin: hemopexin-like; T: FLAG tag.

Figure 3B: A representative zymogram depicting a gelatinolytic band for leukolysin. Control (lane 1) and MMP25cat (lanes 2-7) transfected MDCK cells were stable clones and serum free supernatants from these clones were collected and analyzed by zymography as described (Pei 1996). Out of 6 clones shown, only three (lanes 3, 4, and 6) are positive for leukolysin$_{1-280}$. The high molecular weight gelatinolytic species is the endogenous MMP9 from MDCK cells. Clone #17-13 was chosen for further study in panel C.

Figure 3C: Analysis of intracellular and secreted leukolysin: Control (lanes 2 and 4) and MMP25cat17-13 (lanes 3 and 5) were grown to confluency and washed three times with PBS before replenished with serum free media. 48 hrs later, conditioned media were collected and the cells were lysed in RIPA buffer. The supernatants (lanes 1, 2) and cell lysates (lanes 3, 4) were analyzed by Western blotting using M2 antibody as described previously (Pei 1999a). The pro- species is indicated by arrow and the active one by an arrowhead.

Figure 3D: Characterization of the purification process. Conditioned media from clone #17-13 (150 ml, lane 3, 6) were loaded onto an M2-agarose column. The unbound materials (lanes 3 and 7), washes (lanes 4, 8 and 10) and eluted samples (lanes 5, 9, 11) were analyzed by Western blotting using M2

antibody (lanes 2-5), zymography (lanes 6-9) and staining with brilliant blue R250 (lanes 10-11). It is estimated that ~100 ng of the eluted materials were loaded in lane 11, 20 ng for lanes 5, 9 respectively.

Figure 3E: The purified leukolysin is an active enzyme. Denatured Type I collagens (5 μg, lanes 1-3), were incubated either alone (lanes 1) or with purified leukolysin (20 ng, lanes 2, 3) in the absence (lanes 2) or presence (lanes 3) of synthetic inhibitor BB94 (5 μM) for 8 h at 37°C (lanes 4-6) and analyzed by SDS-PAGE as described in Materials and Methods in Example 1.

Figure 4: Proleukolysin in secretory granules. Figure 4A: Schematic diagram of leukolysin-GFP construct. The structure of leukolysin is shown on the upper portion. Below is the in-frame fusion between leukolysin$_{1-280}$ and GFP tagged with FLAG (T) (see Materials and Methods in Example 1) designed to track the localization of proleukolysin inside the cells.

Figure 4B: A representative zymogram of stable clones transfected with MMP25cat-GFP. Control (lane 5) and leukolysincat-GFP transfectants (lane 1-4) were grown to confluency and conditioned with serum free media which were subsequently analyzed as described in Figure 3. GFP contributes about 22 kDa to the molecular weight of the fusion protein (50 vs. 28 kDa, see Figure 5).

Figure 4C: Confocal localization of MMP25cat-GFP. The left panel is confocal image of control transfected cells from lane 5 of Figure 4B. The right panel is for cells transfected with MMP25cat-GFP (clone 15-11, lane 4 of Figure 4B) sectioned optically on a BioRad MRC confocal system. The intracellular proleukolysin is localized mainly in secretory granules throughout the cell bodies.

Figure 5: Leukolysin/MT6-MMP/MMP25 in PMNs. Figure 5A: RT-PCR analysis of leukolysin (MMP25) among subpopulations of leukocytes. Total RNAs (5 μg) isolated from lymphocytes (lymphs, lanes 3), PMNs (lane 4) and monocytes (monos, lane 5) were reverse-transcribed and PCR amplified as described (Pei 1999c) for leukolysin (35 cycles, upper panel) or the internal reference gene GAPDH (25 cycles, lower panel).

Figure 5B: Northern blot. Total RNA (20 μg) from PMNs was fractionated on a 1.0 % agarose gel, blotted onto Nylon membrane and probed

with ^{32}[P] labeled leukolysin specific probe for 16 h. After washing, the filter was exposed to a phospho-image screen and scanned on an ABI imager. Note that the size of leukolysin mRNA is ~4 kb.

Figure 6: Generation of anti-leukolysin antisera. Figure 6A: Schematic presentation of leukolysin domain structure and the regions from which two peptides were synthesized as indicated. S: signal peptide, Pro: prodomain, R: furin cleavage site, CAT: catalytic domain, H: hinge region, Pexin: hemopexin-like domain, G: putative GPI anchor.

Figure 6B: Screening for anti-leukolysin antisera. Sera samples from 5 different guinea pigs from A to E were prepared and used for immunoblotting against cell lysates from MDCK (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19) or MDCK transfected with a full-length leukolysin expression construct, pCR3.1MT6 (Pei 1999c) (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20) either alone (lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20) or pre-absorbed with the immunizing peptides (lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18). The major leukolysin species are marked with arrows.

Figure 7: Specificity of anti-leukolysin antisera. Figure 7A: Schematic illustrations of expression constructs for MT1, 3, 4, 5, 6-MMPs and CA-MMP. The domain structures were arranged and marked as described in Fig. 2. F: FLAG epitope; C.A.: cysteine-array.

Figure 7B: Anti-leukolysin antisera is specific to leukolysin. MDCK cells transfected with either control vector (lanes 1, 8) or MT1-MMP (lanes 2, 9), MT3-MMP (lanes 3, 10), MT4-MMP (lanes 4, 11), MT5-MMP (lanes 5, 12), CA-MMP (lanes 6, 13) or MT6-MMP (lanes 7, 14) were extracted with 1% Triton in PBS and analyzed by western blottings with anti-FLAG antibody (lanes 1, 3, 4, 5, 6, 7) or anti-MT1 antibody (lane 2), or anti-leukolysin antisera (lanes 8-14). The immunoreactive species were detected using alkaline phosphatase conjugated secondary antibody as described (Pei 1998). * indicates that anti-MT1-MMP antibody was used for MT1 protein in lane 2. The leukolysin product is indicated by an arrow.

Figure 8: Identification of leukolysin in neutrophil lysates. Neutrophils (10^5 cells/lane) were lysed in 1% Trition X-100 (PBS) and analyzed by SDS-PAGE (lane 1), and western blotting without (lanes 2) or with (lane 3)

the blocking peptide. The arrow indicates the full-length leukolysin and the arrowheads depict its degraded products.

Figure 9: Subcellular localization of PMNs. Resting PMNs were treated with proteinase inhibitors, disrupted and fractionated on a Percoll gradient as described in Materials and Methods in Example 2 into four fractions α, β1, β2 and γ. Each fraction was assayed for markers in the azurophil granules (MPO, **Figure 9A**), specific granules (NGAL and Lactoferrin, **Figures 9B and 9C**), gelatinase granules (Gela, **Figure 9D**), secretory vesicles and plasma membranes (HAS, HLA, **Figures 9E and 9F**) and total protein contents (**Figure 9G**). **Figure 9H:** Quantification of leukolysins detected in Figure 9I and expressed as percentages of leukolysin in each band over that of the whole cells. **Figure 9I:** Detection of leukolysin in subcellular fractions. Equivalent proportions of proteins from the postnuclear supernatants (S1, lanes 1, 6) as well as the fractionated bands, α (lanes 2 and 7), β1 (lanes 3 and 8), β2 (lanes 4 and 9) and γ (lanes 5 and 10) were loaded onto SDS-PAGE, separated by electrophoresis, transbloted onto PVDF membrane and developed with G280e alone (lanes 6-10) or G280e preincubated with the immunizing peptide (lanes 1-5). Note the complete blockade of anti-leukolysin antibody with the immunizing peptide (lanes 1- 5 vs. 6-10).

Figure 10: Leukolysin on plasma membrane. Figure 10A: Separation of secretory vesicles from plasma membranes. The γ band was further fractionated into secretory vesicles (filled) and plasma membrane (open) by free-flow electrophoresis, and analyzed for HLA (left), latent alkaline phosphatase (AP, middle) and total protein (right). Note that the plasma fraction is free of latent AP, a marker for the secretory vesicles. HLA should be in both the plasma membrane and secretory vesicle as a membrane protein. PM: plasma membrane; SV: secretory vesicles.

Figures 10B and 10C: Equal volumes (20 μl) from the PM (lane1) and SV (lane 2) were analyzed by Western blot for leukolysin as described in Fig. 6. The blot in Figure 10B was quantified by densitometry and presented in Figure 10C as percentages of total leukolysin in γ band without (left column) or with

(right column) normalization against the amount of proteins in each fraction shown in A (on the right column).

Figure 10D: Release of leukolysin by GPI-PLC from resting PMNs. Resting PMNs were divided into two equal aliquots (~2 millions each in 100µl PBS) and treated either alone (lanes 1, 3) or with 0.5 units of GPI-PLC (lanes 2, 4) at 37 °C degree for 2 hrs. The reactions were terminated by pelleting down the cells. The supernatants were analyzed by Western blotting without (lanes 1, 2) or with blocking with the immunizing peptide (lanes 3 and 4). Note leukolysin as a main species at ~55 kDa and minor 50 kDa species indicated by arrow and arrowhead respectively.

Figure 11: PMA stimulated release of leukolysin by PMNs: Equal numbers of PMNs were either treated alone (resting, open) or with PMA for 15 min (activated, filled). Both samples were then fractionated and analyzed as described in Fig. 9. **Figures 11A** to **11G** were measurements for various markers, MPO (**Figure 11A**), NGAL (**Figure 11B**), Lactoferrin (**Figure 11C**), Gelatinase B (**Figure 11D**), albumin (**Figure 11E**), HLA (**Figure 11F**) and total proteins (**Figure 11G**) for the fractionation process as described in materials and methods (Kjeldsen 1994). **Figure 11H** shows the distributions of leukolysin as quantified from gels in panel I. **Figure 11I**: Subcellular distribution of leukolysin in activated PMNs. The fractions, α (lanes 1 and 2), β1 (lanes 3, 4), β2(lanes 5, 6), γ (lanes 7, 8) and supernatants (lanes 9, 10) from both resting (lanes 1, 3, 5, 7) or activated (lanes 2, 4, 6, 8) PMNs were analyzed for leukolysin by Western blot as described in Fig. 9. The arrow indicates the major leukolysin species while the arrowheads for degraded products.

Figure 12: Cytokine and chemokine stimulated secretion of leukolysin from PMNs: PMNs ($4x10^6$cells in 100µl) were incubated either alone (lanes 1, 5) or with IL-1α (100ng/ml; lanes 2, 7), IL-1β (100ng/ml; lanes 3, 7) and IL-8 (100ng/ml; lanes 4, 8) at 37 °C for 15min. The cells were then lysed in 1% Triton X-100 in PBS (100µl). The supernatants (lanes 5-8) and lysates (lanes 1-4) were analyzed for MMP-9 (**Figure 12A**) or leukolysin (**Figure 12B**) by western blotting using anti-MMP-9 or anti-leukolysin antibodies. The arrows

indicate the full-length molecules for MMP-9 and leukolysin. The horizontal bar depicts the novel processed species for leukolysin.

Figure 13: A model for the distribution and secretion of leukolysin in neutrophils. Various granules and vesicles were indicated in the boxes on the lower left corner. The stored leukolysin is discharged from granules when stimulated by cytokines or chemokines such as IL-8. The insert on the lower right corner depicts the shedding of leukolysin enzymatically by GPI-PLC treatment or PMA treatment with a yet to identified mechanism. PMA: phorbol myristate acetate; PLC: GPI-PLC.

Figure 14: Leukolysin in cells implicated in asthma: Expression of leukolysin in eosinophils and mast cells.
RNAs were isolated from neutrophils (lane 1), eosinophils (lane 2), mast cells (lane 3) and macrophage cell line KU-182, reverse transcribed and PCR amplified for leukolysin (upper) and the internal reference gene GAPDH. Note that eosinophils may express the highest amount of leukolysin due to the less amount of RNA used judged from the GAPDH levels (upper and lower part of lane 2). Surprisingly, mast cells are also sources of leukolysin (lane 3)

Figure 15: Expression and regeneration of recombinant leukolysin in *E. coli*. The catalytic domain (aa107-280) was cloned into a modified pET15b vector (pET15bSma1) and the resultant plasmid was transformed into DE3 cells for expression. The cells were lysed by sonication and leukolysin protein was purified in the inclusion body. From lane 1 to 5, the purified inclusion body from the induced DE3:pET15b Sma1 -Leukolysin were sequentially diluted in TC buffer (50mM Tris pH 7.5, 5mM CaCl2, 10uM ZnCl, 1% Triton X-100). Lane 6 was a direct dilution of the purified protein in TC buffer in a 1:81 ration. Lanes 7, 8, 9 are similar dilution as in lane 6, but at different time. Note that the purified materials can be refolded to generate proteolytic activity (white band). The arrow indicates the purified leukolysin. The arrow head indicates the main proteolytic species from the purified one.

Figure 16: Demonstration that the refolded leukolysin can cleave substrates in solution. While zymography in Fig. 15 can detect any proteolytic species whether they are latent or active, it is necessary to ascertain that the

refolded leukolysin is active. To accomplish that, a solution assay was adopted to show that three different preparations of leukolysin (lanes 3-5) can all degrade the substrate (gelatin, lane 1) efficiently in a 12 h incubation period. Further more, MMP inhibitors such as synthetic BB94 or natural TIMP1 and 2 (lanes 7-10) can completely block its activity.

Detailed Description

A novel matrix metalloproteinase (MMP) was identified from peripheral blood leukocytes and found to be specifically expressed by resting neutrophils among 29 different tissues and cell types examined. Named "leukolysin" for its specific expression in leukocytes, it is apparently the sixth member of the membrane-type MMP subfamily (MT6-MMP) and has a serial designation of MMP25 (Pei 1999c). Leukolysin has a potential glycosylphosphatidylinositol (GPI) anchor. Known for their destructive potentials (Weiss 1989), neutrophils may deploy leukolysin when exiting the blood stream to reach inflammatory sites and/or when mediating the destruction of invading pathogens and infected host tissues.

MMP25 encodes for 562 residues with common MMP domains, *i.e.*, pre-, pro-, catalytic-, hinge- and hemopexin-like domains. It also has a RXK/RR motif, known for its role in MMP zymogen activation, and a C-terminal hydrophobic segment. Overall, leukolysin displays the strongest homology to the newly identified MT-MMP subgroup with 45% and 39% identities to MT4- and MT1-MMPs vs. 30% and 31.5% to MMP1 and MMP3 respectively. Unlike MT4-MMP whose proteolytic activity remains to be demonstrated, a C-terminally truncated leukolysin is expressed as a strong gelatinolytic species at 28 kDa on zymograms, which is derived from a cell-associated 34 kDa proenzyme, presumably by furin or proprotein convertase-mediated removal of the propeptide (~6 kDa). Indeed, when purified to homogeneity, the 28 kDa species behaves as a fully active enzyme capable of degrading gelatin efficiently in solution without further activation. By green fluorescent protein (GFP) tagging, the intracellular proenzyme is localized to granules throughout the cell, suggesting that activation occurs immediately prior to secretion. Taken together,

leukolysin may be part of the proteolytic arsenal deployed by neutrophils during inflammatory responses.

Leukolysin expression at the mRNA level was localized to neutrophils only; it was not seen in monocytes or lymphocytes. To understand the biological functions of leukolysin, subpopulations of leukocytes were screened. Mono-specific polyclonal antisera raised against a synthetic peptide derived from its hinge region can recognize a major species at 56 kDa in addition to several minor ones between 38-45 kDa in neutrophil lysates. These leukolysin products are apparently distributed among specific granules, gelatinase granules, secretory vesicles and the plasma membrane of resting neutrophils, a pattern distinct from that of two previously known neutrophil MMPs: MMP-8 and MMP-9. Consistent with its predicted GPI anchor, leukolysin can be released from intact neutrophils by glycosylphosphatidylinositol-specific phospholipase C. Surprisingly, these membrane-associated leukolysin species are released into extracellular milieu by neutrophils stimulated with phorbol myristate acetate, interleukin-1α and β, and interleukin-8, suggesting that leukolysin species function both as membrane-bound as well as soluble enzymes. These results not only establish leukolysin as the first neutrophil specific MT-MMP but also implicate it as a cytokine/chemokine regulated effector during innate immune responses or tissue injury.

Interestingly, the same gene that encodes leukolysin was isolated by Velasco and colleagues and shown to be expressed in brain tumors (Velasco 2000), echoing a theme in MMP biology that a given MMP may be expressed under defined physiological conditions, but dys-regulated transcriptionally in tissues undergoing carcinogenic transformations (Birkedal-Hansen 1993, Nagase 1999, Woessner 1991, Matrisian 1994).

In certain disease states, it is advantageous to stimulate an inflammatory response, and in particular the activation of neutrophils. For example, it is advantageous to administer leukolysin, or a compound to stimulate natural production of leukolysin through means such a gene-therapy or interleukin conjugated with anti-leukolysin antibody or the hemopexin-domain of leukolysin in patients undergoing chemotherapy who have less than optimal amounts of

neutrophils or other granulocytes, or patients who have received bone marrow transplants, or patients with compromised immune systems (such as an HIV infection). In these situations, a compound or composition containing leukolysin is administered. Alternatively, a composition could be administered that simulates *in vivo* production of leukolysin.

In certain disease states, it would be advantageous to reduce or inhibit the inflammatory response. In situations such as autoimmune conditions, familial Mediterranean fever (FMF), acute inflammations where neutrophils are over-reacted and result in tissue damage, inhibitors against leukolysin may slow and/or block the infiltration of neutrophils to the inflammatory sites and thus prevent or lessen injuries. In these situations, a compound or composition could be administered that reduces or inhibits *in vivo* production of leukolysin.

The present invention provides methods and pharmaceutical preparations for use in the treatment of inflammatory conditions such as 1) acute or chronic inflammation caused by neutrophils; 2) autoimmune diseases where many neutrophil proteins are autoantigens; 3) asthma other related immune disorders caused by granulocytes; 4) tissue injury and organ failure mediated by MMP25 expressing cells such as granulocytes; 5) malignant cancers where MMP25 enables tumor cells to invade and metastasize through its ability to degrade extracellular matrix; 6) genetic disorders with defects in neutrophil deficiency; 7) organ transplant patients with episodes of granulocyte infiltrations; 8) organ failure due to attacks by neutrophils or neutrophil-related immune responses; 9) cardiovascular conditions or damages mediated by neutrophils or its discharged contents; 10) lung damage mediated by neutrophils; 11) skin irritations and blister where granulocytes mediate the destruction of connected tissues; 12) allergy associated with eosinophils and mast cells; 13) arthritis; 14) chronic bronchitis; 15) pulmonary emphysema; 16) α-1 anti-trypsin deficiency; 17) cystic fibrosis; 18) idiopathic pulmonary fibrosis; 19) adult respiratory distress syndrome; 21) familial Mediterranean fever; or 22) periodontitis and oral ulcers. These methods and pharmaceuticals are based upon administration of small molecules (drugs) that are modulators of leukolysin, and pharmaceuticals vehicles for administering leukolysin.

Inhibitors of leukolysin include, but are not limited to, catalytically inactive leukolysin or portions of leukolysin such as its hemopexin-like domain; natural inhibitors such as TIMP-1, 2, 3, 4; synthetic inhibitors with similar properties such BB-94; and natural herb compounds which can bind zinc, thus removing the atom from leukolysin to achieve inhibitions. Stimulators of leukolysin include, but are not limited to cytokines or chemokines such as IL1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12; modified version of leukolysin exhibiting heightened activities; G-CSF or GM-CSF, which can upregulate leukolysin expression; and transcriptional factors in the class of C/EBPs, which controls the transcription of myeloid lineage.

Formulations of Compounds

In cases where compounds are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compounds as salts may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids that form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Pharmaceutically acceptable salts are obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids also are made.

The compounds may be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intravenous, intramuscular, topical or subcutaneous routes.

Thus, the present compounds may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or

soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts may be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage

and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders containing the active ingredient that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium containing, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

While it is possible that, for use in therapy, the compounds or compositions of the present invention, or their salts, may be administered as the pure dry chemicals, as by inhalation of a fine powder via an insufflator, it is preferable to present the active ingredient as a pharmaceutical formulation. The invention thus further provides a pharmaceutical formulation having one or more

therapeutic compound, or pharmaceutically acceptable salts thereof, together with one or more pharmaceutically acceptable carriers therefor and, optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Pharmaceutical formulations include those suitable for administration by inhalation or insufflation or for nasal, intraocular or other topical (including buccal and sub-lingual) administration. The formulations may, where appropriate, be conveniently presented in discrete dosage units and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association the active compound with liquid carriers or finely divided solid carriers or both.

For administration to the upper (nasal) or lower respiratory tract by inhalation, the compounds according to the invention are conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may have a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the compounds according to the invention may take the form of a dry powder composition, for example, a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges. Alternatively, it may be provided in gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator or a metered-dose inhaler.

For intra-nasal administration, the compounds of the invention may be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker).

Drops, such as eye drops or nose drops, may be formulated with an aqueous or non-aqueous base also having one or more dispersing agents, solubilizing agents or suspending agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered via a simple eye dropper-capped bottle, or via a plastic bottle adapted to deliver liquid contents dropwise, via a specially shaped closure.

For topical administration to the eye, nasal membranes or to the skin, the compounds according to the invention may be formulated as ointments, creams or lotions, or as a transdermal patch or intraocular insert. Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents.

For topical administration, the present compounds may be applied in pure form, *i.e.*, when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid. Formulations suitable for topical administration in the mouth or throat include lozenges having active ingredient in a flavored base, usually sucrose and acacia or tragacanth; pastilles having the active ingredient in an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes having the active ingredient in a suitable liquid carrier.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

When desired, the above-described formulations adapted to give sustained release of the active ingredient may be employed, *e.g.*, by combination with certain hydrophilic polymer matrices. The pharmaceutical compositions according to the invention may also contain other active ingredients such as antimicrobial agents, or preservatives. The compounds of the invention may also be used in combination with other therapeutic agents, such as bronchodilators or anti-inflammatory agents.

Examples of useful dermatological compositions that can be used to deliver the compounds of the present invention to the skin are known to the art; for example, see Jacquet *et al.* (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith *et al.* (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compounds of the present invention can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

It will be further appreciated that the amount of a compound of the invention required for use in treatment will vary not only with the particular compound selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician. The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.* into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye or nose.

Generally, the concentration of the compound(s) of the present invention in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

Antibodies Specific for Leukolysin

The present invention provides poly- and monoclonal antibodies that specifically bind leukolysin. In particular, the invention provides monoclonal antibodies that specifically bind to the catalytic domain, and thus directly inhibit its catalytic activity; bind to the hinge and hemopexin domain, which can inhibit its ability to catalyze the cleavage of macromolecules by hindrance; bind to the pro-region to prevent activation of leukolysin; or bind to the GPI anchor region to prevent its release from the cell surface.

Alternatively to the conventional techniques for preparing antibodies in laboratory and farm animals, monoclonal antibodies against leukolysin can also be prepared using known hybridoma cell culture techniques. Monoclonal antibodies against leukolysin can be prepared using known hybridoma cell culture techniques. In general, this method involves preparing an antibody-producing fused cell line, *e.g.*, of primary spleen cells fused with a compatible continuous line of myeloma cells, and growing the fused cells either in mass

culture or in an animal species, such as a murine species, from which the myeloma cell line used was derived or is compatible. Such antibodies offer many advantages in comparison to those produced by inoculation of animals, as they are highly specific and sensitive and relatively "pure" immunochemically. Immunologically active fragments of the present antibodies are also within the scope of the present invention, *e.g.*, the F(ab) fragment, as are partially humanized monoclonal antibodies.

It will be understood by those skilled in the art that the hybridomas herein referred to may be subject to genetic mutation or other changes while still retaining the ability to produce monoclonal antibody of the same desired specificity. The present invention encompasses mutants, other derivatives and descendants of the hybridomas.

It will be further understood by those skilled in the art that a monoclonal antibody may be subjected to the techniques of recombinant DNA technology to produce other derivative antibodies, humanized or chimeric molecules or antibody fragments which retain the specificity of the original monoclonal antibody. Such techniques may involve combining DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of the monoclonal antibody with DNA coding the constant regions, or constant regions plus framework regions, of a different immunoglobulin, for example, to convert a mouse-derived monoclonal antibody into one having largely human immunoglobulin characteristics (see EP 184187A, 2188638A).

Immunoassays

The present invention also provides a method for detecting or determining the presence or the level of leukolysin in a sample of physiological fluid containing leukolysin. The types of immunoassays that can be employed to determine the relative or absolute amount of eosinophil peroxidase in a physiological sample include those assay methods, formats and kits disclosed in U.S. Pat. No. 5,516,639.

Analytes may be distinguished from other sample components by reacting the analyte with a specific receptor for that analyte. Assays that utilize

specific receptors to distinguish and quantify analytes are often called specific binding assays. The monoclonal antibodies of the present invention are applicable to a variety of specific binding assay formats. For example, various direct-binding assays may be employed with these reagents. In such assays, receptors, such as antibodies or other binding proteins, are chemically coupled to make a cross-linked protein complex and the complex is immobilized on the solid phase. The immobilized chemically cross-linked protein complexes are contacted with a sample containing the analyte of interest, which may be distinguished from other components found in the sample. For example, an antibody of the present invention can be immobilized on the surface of a solid substrate and used as a capture antibody, which can specifically bind to leukolysin in a physiological fluid. Suitable substrates include particulate substrates such as polystyrene beads, the wells of plastic microtiter plates, paper or synthetic fiber test strips and the like. The immobilized antibody can then be contacted with the test sample to be assayed, *e.g.*, with a physiological fluid such as plasma, serum, tears, urine, whole gut lavage, mucus, feces, GI tract tissue including mucosa and submucosa, jejunal effluent and the like. The resulting antibody-leukolysin binary complex can then be contacted with an anti-leukolysin antibody, such as rabbit anti-leukolysin serum.

Following binding of the analyte by the immobilized complex, the solid phase may be washed and then contacted with an indicator. The term indicator in the context of this invention means a labeled conjugate. The conjugate comprises an antibody, antibody fragment, binding protein or analyte depending on assay format, and the label is a fluorescent, enzymated, colorimetric, radiometric or other labeling molecule that is associated either directly or indirectly with the conjugate. The label may be comprised of an enzymatic compound that produces fluorescence upon contact with a substrate. The extent to which the indicator is present on the solid support can be correlated with the amount of unknown analyte (see, for example, Tijssen, 1985).

The anti-leukolysin monoclonal antibody of the present invention can be itself coupled to a detectable label of a binding site for a detectable label. For example, the antibodies can be labeled radioisotopically, *e.g.*, by ^{125}I, or

conjugated directly to a detector enzyme, *e.g.*, alkaline phosphatase or horse radish peroxidase, or can be labeled indirectly with a binding site for a detectable label, *e.g.*, via biotinylation. The biotinylated antibody can then be detected by its ability to bind to a an avidin-linked enzyme. If the second antibody is biotinylated, a detector enzyme conjugated to avidin will be subsequently added. The final step for detecting enzymes conjugated to monoclonal antibody or to avidin is the addition of a substrate appropriate for the enzyme to allow quantitative colorimetric detection of reaction product. The value (read in optical density units) can be converted to fmol of leukolysin by reference to a standard curve generated in a control assay in which a standard extract of detergent-solubilized leukolysin is added in graded concentrations to the immobilized anti-leukolysin monoclonal antibody.

The antibodies of the present invention can be used in many other assay formats, such as competitive immunoassays, bead agglomeration assays and sandwich-type immunoassays, such as ELISA, as would be recognized by the art.

In competitive assay formats, the solid phase containing immobilized chemically cross-linked protein complexes with specificity for a selected analyte is contacted with a sample presumably containing such analyte and with a specific competitive reagent. The specific competitive reagent may be a labeled analog of the analyte. In this specific embodiment, the labeled analog competes with the sample analyte for binding to a receptor immobilized on the solid phase.

In the alternative, an analyte may be coupled to a solid phase and contacted with a sample and with a specific competitive cross-linked protein reagent, for example, a labeled receptor for the analyte. In this format, sample analyte competes with solid phase analyte for binding with soluble labeled cross-linked receptor. In both embodiments, the amount of label bound to the solid phase after washing provides an indication of the levels of analyte in the sample. That is, the amount of analyte in a sample is inversely proportional to the amount of analyte in the sample.

Another embodiment of the present invention is a diagnostic kit for detecting or determining the presence of leukolysin in a physiological sample.

The present immobilized antibodies and labeled antibodies are conveniently packaged in kit form, wherein two or more of the various immunoreagents will be separately packaged in preselected amounts, within the outer packaging of the kit, which may be a box, envelope, or the like. The packaging also preferably comprises instruction means, such as a printed insert, a label, a tag, a cassette tape and the like, instructing the user in the practice of the assay format.

For example, one such diagnostic kit for detecting or determining the presence of leukolysin comprises packaging containing, separately packaged: (a) a solid surface, such as a fibrous test strip, a multi-well microliter plate, a test tube, or beads, having bound thereto antibodies to leukolysin; and (b) a known amount of antibodies specific to leukolysin, wherein said antibodies comprise a detectable label, or a binding site for a detectable label.

Physiological Fluids

Physiological samples from patients include plasma, serum, tears, urine, whole gut lavage, lung lavage, peritoneal lavage, skin blister fluids, mucus, feces, GI tract tissue including mucosa and submucosa, jejunal effluent and the like.

Thus, the present invention provides methods for measuring leukolysin in the blood and in the urine as markers of ongoing inflammation for use in the diagnosis of inflammatory disorders. In addition, the assays can be used to determine the anti-inflammatory efficacy of different therapeutic regimens.

An immunoassay test for the total concentration of leukolysin has been developed and the reagents have been or can be produced in mass quantities easily. The invention can be used as a point-of-care diagnostic tool to detect and assist in the management of inflammatory disorders The immunoassay can be performed easily and inexpensively at the patient's bedside. In addition, since the assay detects ongoing inflammation, it is also useful for guiding therapy.

For purposes of more clearly and accurately describing the invention herein, certain terminological conventions have been adopted in the following discussion. These conventions are intended to provide a practical means for enhancing description of the invention, but are not intended to be limiting, and

the skilled artisan will appreciate that other and additional, albeit not inconsistent, interpretations can be implied.

An "analog" or "variant" of leukolysin is a polypeptide that is not completely identical to native leukolysin. Such an analog of leukolysin can be obtained by altering the amino acid sequence by insertion, deletion or substitution of one or more amino acids. The amino acid sequence of the protein is modified, for example by substitution, to create a polypeptide having substantially the same or improved qualities as compared to the native polypeptide. The substitution may be a conserved substitution. A "conserved substitution" is a substitution of an amino acid with another amino acid having a similar side chain. A conserved substitution would be a substitution with an amino acid that makes the smallest change possible in the charge of the amino acid or size of the side chain of the amino acid (alternatively, in the size, charge or kind of chemical group within the side chain) such that the overall peptide retains its spatial conformation but has altered biological activity. For example, common conserved changes might be Asp to Glu, Asn or Gln; His to Lys, Arg or Phe; Asn to Gln, Asp or Glu and Ser to Cys, Thr or Gly. Alanine is commonly used to substitute for other amino acids. The 20 essential amino acids can be grouped as follows: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine having nonpolar side chains; glycine, serine, threonine, cystine, tyrosine, asparagine and glutamine having uncharged polar side chains; aspartate and glutamate having acidic side chains; and lysine, arginine, and histidine having basic side chains. Stryer (1981) p. 14-15; Lehninger (1975) p. 73-75.

It is known that analogs of polypeptides can be obtained based on substituting certain amino acids for other amino acids in the polypeptide structure in order to modify or improve biologic activity. For example, through substitution of alternative amino acids, small conformational changes may be conferred upon a polypeptide which result in increased activity. Alternatively, amino acid substitutions in certain polypeptides may be used to provide residues which may then be linked to other molecules to provide peptide-molecule conjugates which retain sufficient biologic properties of leukolysin. Certain

analogs that are linked to labels or solid phases but retain the ability to bind to anti-leukolysin antibodies, may be used as competitors in competitive immunoassays for leukolysin.

The degree of homology (percent identity) between a native and a variant sequence may be determined, for example, by comparing the two sequences using computer programs commonly employed for this purpose. One suitable program is the GAP computer program described by Devereux (1984), which is available from the University of Wisconsin Genetics Computer Group.

The amino acid sequence of the analog of leukolysin corresponds essentially to the native leukolysin amino acid sequence. As used herein "corresponds essentially to" refers to a polypeptide sequence that will elicit a biological response substantially the same as the response generated by native leukolysin. Such a response may be at least 60% of the level generated by native leukolysin, and may even be at least 80% of the level generated by native leukolysin. A variant of the invention may include amino acid residues not present in the corresponding native leukolysin or deletions relative to the corresponding native leukolysin.

An amino acid is "operably linked" when it is placed into a functional relationship with another amino acid sequence. Generally, "operably linked" means that the amino acid sequences being linked are contiguous.

An "antibody" in accordance with the present specification is defined broadly as a protein that binds specifically to an epitope. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein (1975) and by Campbell (1991); as well as the recombinant DNA method described by Huse *et al.* (1989).

As used herein, the term "monoclonal antibody" (or "mAb") refers to any homogeneous antibody or antigen-binding region thereof that is reactive with, preferably specifically reactive with, a single epitope or antigenic determinant. The term "monoclonal antibody" as used herein may, however, refer to homogeneous antibodies that are native, modified, or synthetic, and can include hybrid or chimeric antibodies. The term does not include "polyclonal

antibodies," as that term is commonly understood. A "polyclonal antibody" is a group of heterogeneous antibodies that all recognize a single epitope or antigenic determinant.

The term "antigen-binding region" refers to a naturally occurring, modified, or synthetic fragment of an antibody of the invention that is reactive with an epitope. Such antigen-binding regions include, but are not limited to, Fab, $F(ab')_2$, and Fv fragments.

Functional equivalents of the antibody of the invention further include fragments of antibodies that have the same binding characteristics as, or that have binding characteristics comparable to, those of the whole antibody. Such fragments may contain one or both Fab fragments or the $F(ab')_2$ fragment. Preferably, the antibody fragments contain all six complement determining regions ("CDRs") of the whole antibody, although fragments containing fewer than all of such regions, such as three, four or five CDRs, may also be functional. Fragments may be prepared by methods well known in the art. Other functional equivalents of the antibody of the invention include other molecules that specifically bind leukolysin, for example, receptors that bind leukolysin and peptides or nucleic acids that have been selected for their ability to bind leukolysin (*e.g.*, by phage display or SELEX methods).

The antibodies of the present invention are specifically reactive with leukolysin. The antibody is not significantly cross-reactive with other polypeptides. Among other properties of the antibody of the invention, it is demonstrated herein that the antibody is reactive with the polypeptide defined by leukolysin (Pei 1999b), or a fragment thereof, such as GKAPQTDYDKPTRKPLA (G280) (SEQ ID NO:1). Proteins containing SEQ ID NO:1, or proteins containing other epitopes of leukolysin within their primary structures, and lacking significant stearic interference from higher order structures will, therefore, bind with one or more of the detecting antibodies or polyclonal antisera of the invention. Such proteins may be naturally occurring or synthetically made, *e.g.*, produced by conventional synthetic or recombinant methods such as are known in the art. Homologs of leukolysin, and proteins containing at least one epitope-defining sequence, are also expected to be

reactive with one or more of the detecting antibodies or polyclonal antisera of the invention. However, the antibodies and antisera exhibit no substantial cross-reaction with moieties lacking this epitope. Peptides or proteins containing this epitope can be detected using the immunoassay of the invention, can be used as calibrators or standards, or can be labeled or immobilized and used as competitors in immunoassays for leukolysin.

Generally, to be useful as an immunogen, a peptide fragment must contain sufficient amino acid residues to define the epitope of the molecule being detected. If the fragment is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet hemocyanin and bovine serum albumin. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a thiol-reactive moiety on the carrier molecule such as a cysteine residue or a maleimide group.

The present invention provides animals that produce polyclonal antibodies reactive with an epitope of leukolysin. The invention also provides hybridoma cell lines that produce monoclonal antibodies reactive with an epitope of leukolysin. The antibodies produced by these animals and hybridomas are also important aspects of the invention.

The hybridoma technology originally described by Kohler and Milstein (1975) can be used to prepare hybridoma cell lines whose secretory product, monoclonal antibodies, are reactive with an epitope or antigenic determinant of leukolysin. A general method of preparing hybridoma cell lines of the invention is described below. Those skilled in the art will recognize that the present invention, including the monoclonal antibodies and hybridoma cell lines described in detail herein, provide a variety of ways to make the hybridomas, and thus the antibodies of the invention. Hybridoma cell lines of the invention can be prepared using leukolysin, or an epitope of leukolysin for activation of immunologically relevant spleen cells. Generally, a host mammal is inoculated with a peptide or peptide fragment as described above, and then boosted. Spleens are collected from inoculated mammals a few days after the final boost. Antibody-producing spleen cells are then harvested and immortalized by fusion

with mouse myeloma cells. The hybrid cells, called hybridomas, are continuous cell lines resulting from the fusion, which are then selected and screened for reactivity with the peptide. The artisan is referred to Kohler and Milstein (1975); Kennett *et al.* (1980); and Goding (1986) for further details on hybridoma technology. *See also* Campbell (1991).

The specific anti-leukolysin antibodies described herein are merely illustrative of the invention, and all antibodies that are specifically reactive with leukolysin, regardless of species of origin or immunoglobulin class or subclass designation, including IgG, IgA, IgM, IgE, and IgD, are included in the scope of this invention. The present invention also provides antigen-binding fragments of the anti-leukolysin antibodies.

As discussed above, antibodies of the invention can be constructed and isolated by immunization of animals, preparation of hybridomas, and identification of antibodies with a reactivity to leukolysin described. Antibodies of the invention can be identified also by immunoprecipitation and competitive binding studies using the antibody produced by the cell lines described herein.

Immunoprecipitations using the anti-leukolysin monospecific antibody can be used to determine antigenic identity. Confirmation of identity can be obtained by depleting the antigen from testable samples such as plasma samples, using excess amounts of one anti-leukolysin antibody and observing the inability of another antibody to immunoprecipitate leukolysin from the treated sample. Also, in instances in which the antibodies bind with the same epitope or closely associated epitopes, each antibody will compete with the other(s) for binding to leukolysin. Competitive binding studies are generally known in the art, and one conventional type is presented in the examples below.

Treatment of antibody preparations with proteolytic enzymes such as papain and pepsin generates antibody fragments, including the Fab and $F(ab')_2$ species, that retain antigen-binding activity. Treatment of the antibodies of the invention with such enzymes can therefore be used to generate leukolysin antigen-binding fragments of the invention. The preparation of antigen-binding fragments of the antibodies of the invention and their diagnostic and therapeutic usefulness, as well as other applications, suggest themselves to the skilled

artisan. Antigen-binding fragments of the anti-leukolysin antibody are especially useful in therapeutic embodiments of the present invention.

Those skilled in the art will recognize that the antigen-binding region of the antibodies and antibody fragments of the invention is a key feature of the present invention. The anti-leukolysin hybridoma cells of the invention serve as a preferred source of DNA that encodes such antigen-binding regions of the invention. This DNA, through recombinant DNA technology, can be attached to DNA that encodes any desired amino acid residue sequence to yield a novel "hybrid," or "chimeric," DNA sequence that encodes a hybrid, or chimeric, protein. In such a fashion, chimeric antibodies of the invention, in which one portion of the antibody is ultimately derived from one species and another portion of the antibody is derived from another species, can be obtained. However, the present invention also comprises any chimeric molecule that contains an leukolysin antigen-binding region.

Antibodies of the present invention can also be labeled by conjugation to any detectable group, such as fluorescent labels, enzyme labels, and radionuclides to identify expression of leukolysin, or cleavage products including leukolysin or parts thereof. Suitable detectable labels may be selected from among those known in the art, including, but not limited to, radionuclides, enzymes, specific binding pair components, colloidal dye substances, fluorochromes, reducing substances, latexes, digoxigenin, metals, particulates, dansyl lysine, antibodies, protein A, protein G, electron dense materials, chromophores, electrochemiluminescent substances, chemiluminescent substances, electroactive substances and the like. Effectively, any suitable label, whether directly or indirectly detectable, may be employed. One skilled in the art will clearly recognize that these labels set forth above are merely illustrative of the different labels that could be utilized in this invention.

Methods for labeling antibodies are well known in the art. For example, methods for labeling antibodies have been described in U.S. Pat. Nos. 3,940,475 and 3,645,090.

The label may be radioactive, *i.e.*, contain a radionuclide. Some examples of useful radionuclides include ^{32}P, ^{125}I, ^{131}I, ^{111}In, and ^{3}H. Use of

radionuclides have been described in U.K. patent document No. 2,034,323, U.S. Pat. Nos. 4,358,535, and 4,302,204.

Some examples of non-radioactive labels include enzymes, chromophores, atoms and molecules detectable by electron microscopy, and metal ions detectable by their magnetic properties.

Some useful enzymatic labels include enzymes that cause a detectable change in a substrate. Some useful enzymes and their substrates include, for example, horseradish peroxidase (pyrogallol and o-phenylenediamine), beta-galactosidase (fluorescein beta-D-galactopyranoside), and alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). The use of enzymatic labels have been described in U.K. 2,019,404 and EP 63,879.

Useful chromophores include, for example, fluorescent, chemiluminescent, and bioluminescent molecules, as well as dyes. Some specific chromophores useful in the present invention include, for example, fluorescein, rhodamine, Texas red, phycoerythrin, umbelliferone, luminol and luminescent bipyridyl or phenanthrolyl containing complexes of ruthenium or osmium..

The labels may be conjugated to the antibody probe by methods that are well known in the art. The labels may be directly attached through a functional group on the probe. The probe either contains or can be caused to contain such a functional group. Some examples of suitable functional groups include, for example, amino, carboxyl, sulfhydryl, maleimide, isocyanate, isothiocyanate. Alternatively, labels such as enzymes and chromophoric molecules may be conjugated to the antibodies by means of coupling agents, such as dialdehydes, carbodiimides, dimaleimides, and the like.

The label may also be conjugated to the antibody probe by means of a ligand attached to the probe by a method described above and a receptor for that ligand attached to the label. Any of the known ligand-receptor combinations is suitable. Some suitable ligand-receptor pairs include, for example, biotin-avidin or -streptavidin, and antibody-antigen. The biotin-avidin combination is preferred. Thus, the anti-leukolysin antibodies of the invention can be derivatized by conjugation to biotin, and used, upon addition of species of

avidins that have been rendered detectable by conjugation to fluorescent labels, enzyme labels, radionuclides, electron dense labels, substrates, etc., in a multiplicity of immunochemical and immunohistological applications.

The antibodies of the invention may also be attached or bound to substrate materials according to methods known to those skilled in the art. Such materials are generally substantially solid and relatively insoluble, imparting stability to physical and chemical disruption of the antibodies, and permitting the antibodies to be arranged in specific spatial distributions. Among substrate materials, materials may be chosen according to the artisan's desired ends, and include materials such as gels, hydrogels, resins, beads, magnetic particles or beads, nitrocellulose, nylon filters, microtiter plates, culture flasks, polymeric materials, and the like, without limitation.

The antibodies of the present invention, whether labeled or unlabeled, can be used in immunological assays to determine the presence of leukolysin in tissue samples from human or animal subjects. Fluid samples of subjects, such as plasma or urine, as well as samples from blood banks, can be evaluated for the presence of leukolysin using an anti-leukolysin antibody of this invention. Moreover, suitable pharmaceutical preparations according to the invention may be employed for *in vivo* use, such as for the visualization of leukolysin or leukolysin-containing substances and structures in a living subject.

Thus, the invention provides a method for binding leukolysin by means of the anti-leukolysin monospecific antibody. Accordingly, leukolysin, natural, modified, and synthetic variants thereof, as well as fragments thereof, may be detected and measured by means of the antibodies of the invention.

In the leukolysin binding method of the invention, the method includes contacting a testable system, in which the presence or absence of leukolysin is to be determined, with a composition comprising an anti-leukolysin antibody or antigen-binding region thereof. The method then involves measuring an amount of specific association or binding between an analyte of the testable system and the antibody. In this method, specific binding of the antibody in the system indicates the presence of the analyte, *i.e.*, leukolysin or leukolysin-containing fragments thereof in the system.

The present invention further provides a method of detecting the presence of leukolysin in a sample. The method involves use of a labeled probe that recognizes protein/peptide present in a biological sample such as a blood or urine sample. The probe may be an antibody according to the invention that recognizes leukolysin-containing analytes present in the sample. Other testing methods known in the art can be adapted to use the antibody of the invention.

A typical method involves the differential separation of degradation products, such as separation of the products by ultracentrifugation. The products are then measured by contacting the products with antibodies that are specifically reactive with or specifically associate with one or more domains of leukolysin. Preferably, such antibodies are specifically reactive with a single degradation product, thereby permitting characterization of the product in relation to other products.

In one embodiment, the detection method employs a anti-leukolysin antibody that has been detectably labeled with a marker moiety. In other embodiments, the method may employ an antibody of the invention that has been bound to a substrate material. In the method, the composition may also include other reagents such as other antibodies that differentially detect other leukolysin subunits or subtypes. This method can be further adapted for use with at least one other antibody having specificity for alternative fragments, permitting differential analysis or characterization of free leukolysin or of leukolysin-containing fragments and other fragments in the same sample. For example, two or more antibodies conjugated to distinct fluorescent labels can be employed as probes in protein separations or other immunometric techniques.

The leukolysin binding method of the invention includes methods known in the art that employ antibodies to bind target substances specifically. Preferred methods include immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA) methods, western blot, immunonephelometry methods, agglutination methods, precipitation methods, immunodiffusion methods, immunoelectrophoresis methods, immunofluorescence methods, radioimmunoassay methods, surface plamon resonance, and immunoassay methods based on the detection of chemiluminescent , fluorescent,

phosphorescent, electrochemiluminescent, bioluminescent or electroactive compounds.

Assays for detecting the presence of proteins with antibodies have been previously described, and follow known formats, such as standard blot and ELISA formats. These formats are normally based on incubating an antibody with a sample suspected of containing the protein and detecting the presence of a complex between the antibody and the protein. The antibody is labeled either before, during, or after the incubation step. The protein is preferably immobilized prior to detection. Immobilization may be accomplished by directly binding the protein to a solid surface, such as a microtiter well or bead, or by binding the protein to immobilized antibodies.

Methods for conducting immunoassays are well known. Techniques that can be used include direct binding formats and competitive binding formats. One common example of the direct binding format is the sandwich binding assay. In a typical example of a competitive assay for leukolysin, leukolysin in a sample competes with labeled leukolysin (or an analog thereof) for binding to a labeled anti-leukolysin antibody. In some common "solid phase" binding assay formats, the label one of the two labeled species is a solid phase support or a capture moiety that is used to bring the reagent onto a solid phase support. Examples of appropriate immunoassay techniques may be found in the Immunassay Handbook, Wild D., Editor, Stockton Press: New York, 1994.

The invention further includes a method for determining or diagnosing the existence of inflammatory disease in a subject. In this method, leukolysin is measured by means of a composition including an anti-leukolysin antibody of the invention. The measured amount of the leukolysin analyte is compared with an amount of leukolysin that is recognized or known to be associated with the inflammatory disease. The method then involves the determination from the measured and standard value(s) of leukolysin the presence or likelihood of inflammatory disease in the subject. The method can include measuring or detecting leukolysin peptides *in vivo*, such as by imaging or visualizing the location and/or distribution of leukolysin, in the body. Alternatively, the method

includes obtaining a medical sample from the subject and measuring leukolysin *ex vivo* or *in vitro*.

The following examples are intended to illustrate but not limit the invention.

EXAMPLES

Example 1: Identification and Characterization of Novel MMP

Materials and Methods

Cell lines and reagents - Mammalian cells including MDCK cells and COS 7 were cultured as described (Pei 1998, Pei 1999b). Reagents associated with recombinant DNA techniques were purchased from Promega (Madison, WI). The RT-PCR / RACE kit was from Roche Diagnostics (Indianapolis, IN). Oligonucleotide primers were made by either the University of Minnesota microchemical core facility or IDT (Coralville, IA). FLAG peptide, anti-FLAG M2 monoclonal antibody and agarose beads were purchased from Sigma (St. Louis, MO).

Molecular cloning and sequence analysis of human leukolysin/MMP25 - Initially, EST clones (AI150017 and AA830519, both from IMAGE consortium (Lennon 1996)) were identified through a BLAST search to encode a putative MMP showing 60% identity to L235-G298 of MT4-MMP (Puente 1996, Kajita 1999). Based on these sequences, subsequent RT-PCR and RACE reactions using RNA isolated from human leukocytes were performed to isolated a composite 2.6 kb cDNA which encodes an open reading frame with a conserved initiation codon and stop codon. Sequencing and sequence analysis were performed as described previously (Pei 1999b).

Tissue distribution of leukolysin/MMP25 - Human cDNA panels were purchased from Clontech (Palo Alto, CA) and amplified for the expression of leukolysin/MMP25 with two primers (TAC GCT CTG AGC GGC AGC (SEQ ID NO:2) and CCC ATA GAG TTG CTG CAG (SEQ ID NO:3)) following this conditions: 2 min at 94°C for denaturation, 35 cycles of 10 sec at 94°C for denaturation, 30 sec at 52°C for annealing and 30 sec at 72°C for extension, followed by 10 min extension at 72°C. The house-keeping gene GAPDH was amplified to estimate the relative amount of cDNA used in each reaction as

described previously (Pei 1999a). Peripheral blood leukocytes were separated into three pools: monocytes, lymphocytes and neutrophils (provided by Mary Dietz of R&D systems, Minneapolis). Total RNA isolated from each fraction was reverse transcribed and amplified as described above. For northern blot analysis, total RNA isolated from neutrophils were fractionated on 1.2% agarose gel at 5, 10 and 20 μg per lanes, transferred to Nylon membrane, stained with methylene blue and hybridized with ^{32}[P] labeled full-length leukolysin/MMP25 cDNA as described (Sambrook 1989).

Expression vectors for leukolysin$_{1-280}$, leukolysin$_{1-280}$-GFP and generation of their stable transfectants - The first 280 residues of leukolysin was isolated by high-fidelity PCR with pfu polymerase (15 cycles) and inserted into two vectors: pCR3.1-FLAG and pCR3.1-GFP-FLAG (Pei, et al unpublished). The 3' end of the insert was designed such that it is inserted in-frame with FLAG and GFP-FLAG respectively. The resulting vectors were sequenced and confirmed to be error-free and fused in-frame as designed. The resulting expression vectors were transfected into MDCK cells and stable clones were obtained by G418 selection (Pei 1998). More than 20 clones were isolated for each transfection and expanded. To screen for positive clones, serum-free conditioned media from each clone were analyzed by gelatin-zymography and western blot using M2 antibody (Pei 1999a). For analysis of cell associated recombinant protein, cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 10 μM leupeptin, 0.1 μM 5-APMSF, 1 mM aprotinin). Cleared of any debris by microcentrifugation, the lysates were fractionated on SDS-PAGE and western blotted with M2 antibody as described above.

Purification and characterization of leukolysin$_{1-280}$ - A stable clone (#17-13) was chosen for micro-scale purification for its relative high yield of the recombinant protein leukolysin$_{1-280}$. Collection of conditioned media and purification by affinity chromatography were carried out essentially as described (Pei 1998). Briefly, 150 ml of conditioned media from cell line 17-13 were obtained from 2 day old culture and cleared of cell debris by centrifugation. An anti-FLAG M2 antibody column (1X1 cm) was then loaded with the cleared

supernatants and washed in Tris-buffered saline (20 mM Tris, pH 7.4, 300 mM NaCl and 5 mM $CaCl_2$). The bound materials were eluted by antigen competition using 2 molar excess of FLAG peptide (Sigma, MO). Fractions were then analyzed by zymography, western blotting and SDS-PAGE stained with brilliant blue R250 as reported previously (Pei 1999a).

Substrate degradation experiments were carried out essentially as described (Pei 1996). Briefly, denatured type I collagen (gelatin, 5 μg) were incubated with purified MMP25 (20 ng) in the activity buffer (150 mM NaCl, 50 mM Tris HCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.1% Brij 35) at 37°C for 8 hrs with or without 5 μM BB94 (British Biotech, UK). The reaction mixtures were analyzed by SDS-PAGE and visualized by brilliant blue R250 staining as described (Pei 1996).

Confocal microscopy - Cells were washed twice in PBS, fixed in 4% paraformaldehyde (30 min) and examined on a BioRad MRC confocal system maintained at the Biological Imaging and Processing Laboratory of the University of Minnesota.

Results

A novel MMP: leukolysin/MMP25/MT6-MMP - Searching the EST database for novel MMP-like genes have resulted in the identification of two latest additions to the MMP family: CA-MMP/MMP23 and MT5-MMP/MMP24 (Pei 1999a, Velasco 1999, Pei 1999b, Llano 1999). Continuing this effective strategy, a putative MMP gene was identified that shares considerable homology to MT4-MMP, which was initially reported by Puente et al (Puente 1996) and recently completed by Kajita and colleagues (Kajita 1999). The full-length open reading frame of this novel MMP was assembled through the EST clones and cDNA fragments isolated by RT-PCR and 5' RACE, and validated by direct amplification of the entire coding region from leukocyte cDNA samples. As shown in Figure 1A, this novel MMP possesses a MMP domain structure such as 1) a cysteine-switch PRCXXPD (SEQ ID NO:4) in the prodomain, 2) a HEXGHXXXXXH (SEQ ID NO:5) zinc binding motif in the catalytic domain and 3) a hemopexin-like domain at the C-terminal portion (Figure 1C).

Interestingly, with a RXR/KR motif between its pro- and catalytic domains, this novel MMP apparently joins a subfamily of MMPs with a well-defined mechanism of zymogen activation mediated by members of the proprotein convertases such as furin in the trans-Golgi network (Pei 1995, Steiner 1998, Nagase 1997). At the C-terminus, a hydrophobic segment is present as a putative transmembrane domain (Figure 1B). Given its origin of isolation and tissue specific expression in neutrophils (See Figure 3), this novel gene was named leukolysin in analogy to stromelysins. Following the MMP serial designation, it was assigned. On the other hand, due to its close relationship to MT4-MMP and the other MT-MMPs, this gene is also called MT6-MMP.

The relationship between leukolysin and other MMPs - As shown in Figure 1B, leukolysin clearly belongs to the MMP family based on its domain structure. To further analyze the relatedness between leukolysin and known MMPs, a multiple sequence alignment was constructed and represented in Figure 1C. Based on the alignment score, leukolysin shows the highest homology to MT4-MMP (45% identity overall), a putative MMP with unknown enzymic and biologic function. In addition, leukolysin has the strongest homology to the rest of the MT-MMP subgroup among all MMPs. In fact, the catalytic domain of leukolysin, the most conserved part of the MMP family, displays 56% amino acid sequence identity to MT4-MMP, compared to 46-48% for MT1, 2, 3, 5-MMPs (Figure 1C). Similarly, the pro- and hemopexin- domains of leukolysin show the closest homology to MT4-MMP when compared to other MT-MMPs (Figure 1C). Based on the alignment, the C-termini are the most divergent and generally fall into two groups. The first group, including MT1, 2, 3, and 5-MMPs, has a traditional transmembrane domain of 24 residues followed by a cytosolic domain with a conserved Valine at their termini (Figure 2a). The second group, made of MT4-MMP and leukolysin, has no visible cytosolic domains after the hydrophobic domains (Figure 1C).

Neutrophils are the primary source of leukolysin/MMP25/MT6-MMP - To investigate the distribution of leukolysin, a panel of cDNAs prepared from 26 tissues and organs were screened for leukolysin expression by PCR. As shown in Figure 2A, peripheral leukocytes are the main source of leukolysin. Bone

marrow and fetal kidney also express minute amounts of leukolysin, probably reflecting residual blood leukocytes in those organs at the time of sample collections. To determine the specific cells responsible for expressing leukolysin, peripheral blood leukocytes were separated into three pools of lymphocytes, neutrophils and monocytes respectively. Total RNA samples were isolated and analyzed for leukolysin expression by RT-PCR. As shown in Figure 2B, leukolysin is exclusively expressed by neutrophils (lane 4), not lymphocytes nor monocytes (lanes 2 and 3). To validate its expression, total RNA from neutrophils were analyzed by northern blot with ^{32}[P] labeled leukolysin cDNA probe. An RNA species migrating around 4.0 kb was detected in as little as 5 μg of total RNA with a short exposure of only 2 hrs in a phosphor-imaging cassette (ABI, MA). Thus, leukolysin is a neutrophil specific MMP.

Expression of leukolysin as a soluble enzyme - As shown in Figure 1, leukolysin possesses a hydrophobic segment which could anchor the protein on cell surface, thus, making purification of the proteinase difficult. Indeed, full-length leukolysin is expressed as cell associated forms (Pei, unpublished data). To circumvent potential problem for purification, a vector was constructed to express leukolysin$_{1-280}$ tagged with FLAG epitope, without the pexin- and putative transmembrane domains (Figure 3A). Since leukolysin contains a furin site between its pro- and catalytic- domains, it is expected that leukolysin $_{1-280}$ will be expressed in latent form in the ER, transported to the Golgi where it is processed into active forms as described for MMP11 and MMP14 (Pei 1995, Santavicca 1996, Pei 1996). The expression vector, pCR3.1MMP25cat, was transfected into MDCK cells and stable clones were isolated and screened by zymography. As shown in Figure 3B, a 28 kDa gelatinolytic species was detected in the conditioned media of several transfectants (lanes 3, 4 and 6). This finding is in sharp contrast to the negative results obtained when similar MT4-MMP was expressed under identical conditions (Trui and Pei, unpublished data). One of the clones with high yields, 17-13 (Figure 3B, lane 3), was chosen for further characterization and purification (see below).

Soluble leukolysin is secreted as a processed and fully active enzyme - With a RRRR motif, leukolysin was expected to be activated by furin or related

PCS intracellularly prior to secretion. Thus, the conditioned media and cell lysates were analyzed by western blot to establish the relationship between extracellular and intracellular leukolysins. As shown in Figure 3C, the conditioned media from 17-13 contains a major immuno-reactive species at ~28 kDa and a minor one at ~34 kDa (Figure 3C, lane 3), potentially corresponding to the active and pro- forms of leukolysin. In contrast, more than 97% of intracellular leukolysin is in the 34 kDa form, compared to ~3% for the 28 kDa active forms (Figure 3C, lane 5), thus validating a precursor/product relationship for the 34 and 28 kDa species. The loss of ~6 kDa is presumably the result of prodomain processing at the RRRR signal by furin or its related PCS (Pei 1995).

To prove that the secreted 28 kDa species is fully active, micro-scale purification was attempted with 150 ml of the conditioned media using a M2 affinity column as described (Pei 1998). As shown in Figure 3D, the FLAG tagged leukolysin (lane 2) was absorbed to the column (lane 3 for input, lane 4 for flow-through) and eluted as single active species (lanes 5, 9, 11) as characterized by SDS-PAGE, Western blotting and zymography (Pei 1999a). As a result, the 28 kDa species was purified to homogeneity as judged by SDS-PAGE (Figure 3D, lane 11). To assay whether the purified leukolysin is active without further activation as predicted, the purified material was incubated with gelatin in the presence and absence of BB94, a synthetic MMP inhibitor. As expected, the 28 kDa species behaves as an active enzyme by cleaving gelatin in a BB94 sensitive fashion (Figure 3E).

Proleukolysin is localized in secretory granules throughout the cell - Since almost all intracellular leukolysin is in latent state (Figure 3C), it must be residing in the trans-Golgi network waiting to be processed by furin or related proprotein convertases (Pei 1995, Steiner 1998). To track the localization of proleukolysin, a GFP fusion construct was engineered to fuse leukolysin$_{1\text{-}280}$ to the green flurorescent protein (GFP) (Figure 4A). Stable cell lines expressing the GFP fusion protein were generated from MDCK cells as described (Pei 1998). More than 15 positive clones were obtained and a few representatives are shown by zymography in Figure 4b. As expected, GFP adds 22 kDa to the secreted gelatinolytic species as a 50 kDa enzyme (Figure 4B). Clone 15-11

(lane 4 of Figure 4B) was selected for further analysis. Western blotting of the supernatants and cell lysates from 15-11 cells demonstrated that the majority of intracellular leukolysin was still in latent form as demonstrated for leukolysin$_{1-280}$ in Figure 3C. To visualize the GFP tagged proleukolysin, confocal microscopy was performed to demonstrate that leukolysin$_{1-280}$::GFP was localized in secretory granules throughout the cell body, contrary to the concentrated localization of perinuclear ER and Golgi network for most secretory proteins. Thus, the signal peptide and the prodomain of leukolysin contain the necessary information to target leukolysin to secretory granules, perhaps relevant to the ones found in neutrophils (Weiss 1989).

Discussion

As a cell type in inflammatory response, neutrophils produce reactive oxidants and proteolytic enzymes to destroy invading pathogens, infected host cells and damaged tissues (Weiss 1989). Leukolysin fits quite naturally into these roles of neutrophils as part of their proteolytic arsenals, perhaps complementing the roles of two MMPs previously found in neutrophils: MMP8 and MMP9 (Hasty 1990, Hibbs 1985). The localization of the C-terminally truncated proleukolysin into the secretory granules of MDCK cells suggests that leukolysin may be stored as zymogens in the granules of neutrophil, a depot of many powerful, yet latent, proteinases (Weiss 1989). Leukolysin is characterized as a potent proteinase against denatured collagen (Figure 3E). Furthermore, a genomic clone has been isolated from mouse SVJ129 genomic library (Yi and Pei, unpublished data).

With a RRRR motif, leukolysin is a new member of a subgroup of MMPs which can be activated by Furin or related proprotein convertases (Hagase 1997). Although the precise mechanism for its activation remains to be investigated, proleukolysin (34 kDa) was shown to be processed into the 28 kDa active species (Figure 3c) presumably by furin or related PCS. MMP activation mediated by furin or related proprotein convertases was first proposed during the characterization of stromelysin-3 (Pei 1995). With the recent identification of leukolysin, MT5-MMP and CA-MMP/MMP23, close to 40% of known MMPs

(8 out of 22) possess a RXK/RR motif, ready to be cleaved and activated by furin or related PCS (Massova 1998, Pei 1999a, Velasco 1999, Pei 1999b, Llano 1999, Nagase 1997). Addition of leukolysin to this group further illustrates the importance of furin- or PC-mediated MMP activation in ECM remodeling processes.

By displaying closest homology to the MT-MMP subgroup, especially MT4-MMP (Figure 1C), leukolysin is also named MT6-MMP. Based on the alignment in Figure 1C, it seems plausible to divide the MT-MMPs into two branches: MT1, 2, 3, 5-MMPs and MT4, 6-MMPs. The defining function for the former group is to activate progelatinase A (Sato 1994, Takino 1995, Pei 1999b), whereas the later remains unknown. The potential mechanism for leukolysin localization is currently under investigation to define the role of the C-terminal hydrophobic domain in membrane anchoring and the roles of signal peptide and prodomain in targeting proleukolysin to intracellular granules (Figure 4). Anchored on cell surface, leukolysin could be in close contact with the underlining substrates and excluded from the anti-proteinase shield in the extracellular milieu, thus, may exert a uniquely powerful force in destroying and remodeling extracellular matrix during neutrophil-mediated inflammatory responses.

Example 2: Subcellular distribution, cytokine- and chemokine- regulated secretion of Leukolysin/MT6-MMP/MMP25 in neutrophils

Materials and Methods

Chemicals, cells, cell culture and immunological reagents - General laboratory chemicals and proteinase inhibitors were from Sigma (St. Louis, MO). Cell culture reagents and fetal bovine sera were from Life Technologies (Rockville, MD). Cell lines including COS, MDCK and its derivatives were obtained and maintained as described (Pei 1999c, Pei 1999b). M2 anti-FLAG monoclonal antibody was purchased from Sigma Chemicals (St. Louis, MO). Anti-leukolysin antisera were raised in guinea pig against synthetic peptides as shown in Figure 6 as described (Pei 1999c). IL-1α, IL-1β, IL-8, and anti-MMP-9 antibody were from R&D system (Minneapolis, MN).

Analysis of MMP25/leukolysin expression - Monocytes, lymphocytes and PMNs were obtained from R&D system (Minneapolis, MN) with ~98% purity. Total RNAs were isolated from these cells with TRI-REAGENT as suggested by the manufacturer (MRC, OH). For RT-PCR analysis, 2 μg of total RNAs were reverse transcribed with superscript II (Life Technologies, MD) in 20 μl reactions. PCR reactions were carried out with 2 μl of RT reactions for both leukolysin and the internal reference GAPDH as described (Pei 1999c). For Northern analysis, total RNA (20μg) from PMNs was denatured with glyoxal and DMSO, fractionated on a 1% agarose gel in 10 mM phosphate buffer (55 V for 5 hr), and then transferred to nylon membrane overnight. The membrane was prehybridized at room temperature for 30 min, hybridized at 62°C for 16-24 hr with ^{32}[P] labeled leukolysin cDNA (~700bp), washed and exposed to an ABI screen, and scanned on a PhosphorImager (ABI, CA).

Characterization of anti-Leukolysin antisera against recombinant MMPs - pCR3.1-MT6, pREP9MT1$_{1-528}$ and pCR3.1MT5$_{1-539}$F were described previously (Pei 1999c, Pei 1996, Wang 1999). pCR3.1MT6$_{1-509}$F was constructed by isolating the cDNA fragment coding 1-509 residues of leukolysin with high fidelity PCR and inserting it into the EcoRV site of pCR3.1. pCR3.1MT3$_{1-556}$F, containing the coding region for residues 1 to 556 with a FLAG tag at its C-terminus, was constructed by isolating the corresponding fragment by PCR and inserting it into pCR3.1 vector as described above. pCR3.1ST3/MT4$_{129-525}$F, harboring a hybrid molecule with the pro-domain of MMP-11 followed by the catalytic, hinge and pexin domains of MT4-MMP, was obtained by inserting a chimera DNA fragment generated through sequential PCR to combine the prodomain of MMP-11 and the mature portion of MT4-MMP as described (Pei 1995). pCR3.1ST3/CA-MMP80-391F, also a chimera between the pro domain of MMP-11 and a fragment from CA-MMP containing predicted matured protein with residues 80 to 391, was constructed by combining the first 80 residues of MMP-11 and a fragment of CA-MMP coding for 80-391 residues. All recombinant constructs were confirmed by sequencing double-stranded DNAs with nested primers. DNA transfection was carried out with lipofectAMINE (Life Technologies, MD) as described (Pei 1999c). Stable

lines were derived from MDCK cells with G418 (400 μg/ml) selection and screened by Western blotting as described (Pei 1998). The recombinant proteins were extracted in 1% Trition X-100 (in PBS). Western blottings were carried out as described using either anti-FLAG antibody M2 or anti-MT1-MMP and G280e (Pei 1999c, Pei 1996). The specificity of antisera was confirmed by pre-absorbing them with the corresponding peptide used for immunization (1μg/ml).

Detection of Leukolysin in PMN lysates - PMN cells (10^7) were extracted with 1ml of 1% Trition in PBS (phosphate buffered saline) with proteinase cocktails (1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aproptin, 5 μM BB94). Cell debris was removed by centrifugation at 15,000g for 15 min at 4°C and the resulting extracts (10μl/lane, ~ 10^5 cells) were analyzed SDS-PAGE and Western blotting.

Subcellular fractionations of PMNs - PMNs (4.5×10^8) isolated from peripheral blood were resuspended in Krebs-Ringer Phosphate (KRP; 130 mM NaCl, 5 mM KCl, 1.27 mM $MgCl_2$, 0.95 mM $CaCl_2$, 10 mM NaH_2PO_4/Na_2HPO_4, pH7.4) + 5mM glucose at 3×10^7 cells/ml and incubated with DFP (5mM) for 15 min. Cells were pelleted by centrifugation and resuspended in 14 ml relaxation buffer and cavitated (Borregaard 1983). 10 ml of the post-nuclear supernatant (S_1) was put on a 3-layer Percoll gradient (generated in relaxation buffer containing 1 mM PMSF) and centrifuged (Kjeldsen 1994). Samples were collected in fractions of 1 ml. Fractions were pooled as α-band (fractions 1-6), β_1-band (fractions 7-12), β_2-band (fractions 13-18) and γ-band (fractions 19-24) (Kjeldsen 1994). These materials were centrifuged to remove Percoll and the pellets were resuspended in 1000 μl PBS. Markers for various granules were assayed as quality control for the fractionation process according to (Kjeldsen 1994). For SDS-PAGE and Western blotting, the samples were boiled in 1x Laemmli sample buffer and stored frozen until assays were performed. Free Flow electrophoresis was carried out to separate the γ-band into plasma membranes and secretory vesicles as described (Sengelov 1992). The markers for plasma membranes and secretory vesicles are HLA and latent alkaline phosphatase respectively and assayed as described (Sengelov 1992). For PMA (phorbol myristate acetate) stimulations, freshly isolated neutrophils were

resuspended in KRP + 5mM glucose at 3 x 10^7 cells/ml and divided in two equal portions (11 ml each). One was kept on ice as control, the other was stimulated at 37° C for 15 min with PMA (2μg/ml). The stimulation was stopped by adding 2 vol of ice-cold buffer (KRP+Glucose). Both the resting as well as the activated portions were pelleted by centrifugation. The supernatants were saved. The pellets from both control and stimulated cells were resuspended in 11 ml buffer and fractionated (see above). The supernatants and fractions were assayed for markers and leukolysin as described above.

Glycosyl-phosphatidylinositol phospholipase (GPI-PLC) treatment of neutrophils - Resting neutrophils (~2x10^7/ml) were incubated with GPI-PLC (0.5 unit) in a 100ul volume for 2 hr at 37°C as suggested by the supplier (Glykomed, Oxford, UK) (Itoh 1999). The supernatants were analyzed by Western blotting as described above.

Treatment of neutrophils with IL-1 α, β and IL-8 - Freshly isolated neutrophils were divided into equal portions (~4x10^6 in 100 μl) and treated with cytokines IL-1 α (100 ng/ml) and IL-1 β (100 ng/ml) and chemokine IL-8 (100 ng/ml) for 15 min at 37°C. The cells were then separated from supernatants by centrifugation (5000 rpm, 15 min) before being lysed with equal volume (100ul) of 1% Trtion X-100 in PBS (see above). Both the supernatants and cell lysates were analyzed for leukolysin and MMP-9 by Western blotting as described above.

Results

Leukolysin is PMN specific - Previously, it was determined that leukolysin is only expressed by pooled peripheral blood leukocytes among 26 different human tissues and cell types (Pei 1999c), in contrast to the subsequent description of apparently the same gene being expressed by brain tumor samples (Velasco 2000). In this Example, experiments were performed to better define the cell population that expresses leukolysin. Toward this end, leukocytes were separated into three main categories of cells, namely lymphocytes, monocytes and polymorphonuclear leukocytes (PMNs). Total RNAs were then extracted for RT-PCR analysis (Pei 1999c). As shown in Fig. 5A, PMNs are the only cells

positive for leukolysin while both lymphocytes and monocytes, known to share MMP profiles with the PMNs, are virtually negative, even with 35 cycles of amplification (lanes 3 and 5 vs. 4). To confirm its presence as mRNA, we performed Northern blot analysis and demonstrated that leukolysin is expressed as a single ~4.0 k mRNA species (Fig. 5B). This message is detectable in as little as 5 μg of total PMN RNA with 3h exposure. Based on these experiments, it was concluded that PMNs are the primary source of leukolysin in peripheral blood.

Generation of polyclonal and mono-specific antisera against Leukolysin - PMNs are cells involved in innate immunity and tissue damage mediated by various enzymes and anti-microbial polypeptides released from intracellular granules (Weiss 1989). Given the finding that leukolysin is PMN specific, it was further hypothesized that leukolysin be part of the proteolytic arsenal stored in those intracellular granules as well. To test this hypothesis, antisera was developed against leukolysin that could detect its protein products specifically. Initially, two peptides, RYALSGSVWKKRTLT (R107) (SEQ ID NO:6) and GKAPQTDYDKPTRKPLA (G280) (SEQ ID NO:1) were designed. The R107 peptide was derived from the beginning of the catalytic domain. Thus, it generated antibody specific for the processed and active leukolysin with a predicted N-terminus at Y108. The second peptide G280 was derived from the hinge region with minimal homology to all other MMPs. Thus, it generated a specific antibody. These two peptides were synthesized with an extra cysteine residue at each N-terminus for conjugation to KLH (Pierce, IL). The immunogenic peptides were injected into a total of 5 guinea pigs (animals A to E) and the immune responses were monitored by collecting blood samples for testing against recombinant leukolysin protein expressed in stable MDCK cell lines (see Materials and Methods above).

As shown in Fig. 6, antisera from R107 were not immunogenic and generated no detectable signals on PVDF membranes immobilized with leukolysin protein (lanes 4 and 8). In contrast, G280 appears to be more immunogenic in guinea pigs and was able to generate immunoreactive antibody in all three guinea pigs injected (arrows, lanes 12, 16, and 20). The antisera from

these animals were able to detect leukolysin, albeit with different specificity and titers. Sera from animals C and D were not suitable due to the nonspecific bands they detected from MDCK lysates that were not blocked by the immunizing peptides (Fig. 6, lanes 11, 12 vs. 9, 10 and 15, 16 vs. 13, 14). However, the sera from animal E appeared to be specific for leukolysin and did not react with any of the MDCK proteins (Fig. 6, lanes 17-20). Designated G280e, its reactivity toward leukolysin (MT6) can be blocked by the immunizing peptides (Fig. 6, lanes 18 vs. 20), a property that would render the antisera ideally suited for the detection of leukolysin protein products in PMN lysates.

Since MMPs, especially MT-MMPs, are highly homologous at the amino acid level, antisera generated for a given member may potentially cross-react with other members. Thus, G280e had the potential to react with other MT-MMPs with similar hinge regions, especially MT4-MMP which shares 56% amino acid identity with leukolysin (Pei 1999c). To test this possibilities, recombinant proteins were produced in MDCK cells for MT1-MMP, MT3-MMP, MT4-MMP, MT5-MMP and CA-MMP and their reactivity toward G280e was tested (see Materials and Methods above). As shown in Figure 7, G280e could only detect recombinant leukolysin (lane 14). Its closest relative, MT4-MMP, was not detected by G280e (Fig. 7, lane 11). Furthermore, G280e did not cross react with recombinant MMP-8 or MMP-9, two MMPs found in neutrophils, under identical conditions. Therefore, G280e is mono-specific for leukolysin.

Detection of leukolysin products in neutrophils - In general, it is more challenging to detect naturally-occurring products of a gene in cells than the over-expressed recombinant forms as described in Figures 6 and 7. Given the specificity G280e exhibited in Figures 6 and 7, an experiment was designed to positively identify leukolysin products from a natural source, *i.e.*, PMNs. In a preliminary experiment, different numbers of PMNs ranging from $9x10^3$, $9x10^4$, $3x10^5$, to $9x10^5$ were lysed in SDS sample buffer and analyzed directly by western blotting. A clear signal was observed in as few as $9x10^4$ cells per lane. Given the recent report that leukolysin may be a GPI-anchored protein (Kojima 2000), freshly isolated PMNs were extracted with 1% Triton in PBS (with

proteinase inhibitor cocktail) for 30 min to harvest all cellular proteins including those from membranes. The total proteome from ~10^5 PMNs was fractionated by SDS-PAGE and detected with brilliant blue R250 staining (Fig. 8, lane 1). The same amount of protein was transferred to a PVDF membrane and probed with G280e. A major 56 kDa species plus a few smaller ones were detected (Fig. 8 lane 2). When G280e were pre-incubated with the immunizing peptides, the signals were completely blocked as expected (Fig. 8, lane 3). It was concluded that neutrophils produce leukolysin protein products, perhaps with the 56kDa species as the full-length molecule and the smaller ones as processed products from the full-length species similar to those reported for MMP-11(Pei 1994).

Distribution of leukolysin in PMN granules - One of the defining features for the PMNs is a series of intracellular granules formed during maturation that store various enzyme systems and anti-microbial agents important for destroying microbial cells and destroying infected tissues (Hampton 1998, Borregaard 1997). MMP-8, the first MMP discovered in neutrophils, is stored in the specific granules (Murphy 1977), while MMP-9 is stored in the gelatinase granules (Borregaard 1997). To define the subcellular distribution of leukolysin, PMNs were fractionated into 4 distinct fractions on a 3 layer Percoll gradient, namely α, β1, β2 and γ potentially representing the azurophil, specific, gelatinase granules, the secretory vesicles and plasma membranes (Kjeldsen 1994). Indeed, these classifications and the fractionation process were validated by assaying known markers established for these granules and vesicles such as myeloperoxidase (MPO) for azurophil granules (Fig. 9A), lactoferrin and NGAL for specific granules (Figs. 9B and 9C), MMP-9 for gelatinase granules (Fig. 9D), albumin for secretory vesicles and HLA for plasma membranes (Figs. 9E and 9F) (Borregaard 1983, Kjeldsen 1994, Borregaard 1997). The distribution of leukolysin was subsequently determined by Western blot analysis (Fig. 9I). S1 represents the postnuclear fractions prior being loaded onto the Percoll gradient and, thus, the total leukolysin proteins (Fig. 9I, lane 1 and 6). Upon fractionation, it became apparent that bulk of leukolysin was in gelatinase granules and secretory vesicles/plasma membrane (Fig. 9I, lanes 9 and 10). A

substantial amount of leukolysin was also present in the specific granules where MMP-8 is localized (Fig. 9I, lane 8), while the azurophil granules are negative (lane 7). The fractionation process preserved the migratory patterns for leukolysin (Fig. 9I, lanes 6 vs. 8, 9, 10) and the signals were specific as judged by the complete blockade with the immunizing peptide on western blots (Fig. 9I, lanes 1-5 vs. 6-10). This broader distribution than those of MMP-8 and MMP-9, argues strongly that leukolysin may play different roles from these two previously identified neutrophil MMPs in neutrophil functions.

Leukolysin is present on plasma membrane - The unexpected identification of leukolysin in the γ band (composed of secretory vesicles and plasma membrane) raised the possibility that it may be on the plasma membrane and displayed on cell surface, a novel location for neutrophil MMPs. To ascertain this possibility, free-flow electrophoresis was used to separate plasma membrane from secretory vesicles (Sengelov 1992). As shown in Fig. 10A, pure plasma membranes were obtained by separating away the secretory vesicles as confirmed by the absence of latent alkaline phosphatase, a marker for secretory vesicles (the middle column). Western blot analysis of these fractions confirms that presence of leukolysin in plasma membrane (Fig. 10B, lane 1), representing ~36% of the leukolysin in γ band (Fig. 10C, left column). Considering the amount of protein in the plasma membrane fraction was considerably less than that of the secretory vesicles (Fig. 10A, right column), it was possible that relative level for leukolysin was similar for both secretory vesicle and plasma membrane. Indeed, when normalized against protein levels, leukolysin appeared to be slightly more concentrated on plasma membrane than in the secretory vesicles (Fig. 10C, right column). Throughout the fractionation process, all the fractions were kept proportional to the total number of cells analyzed (per cell). Thus, the distribution profile of leukolysin in resting PMNs could be estimated as follows: azurophil granules (0%), specific granules (~10%), gelatinase granules (~40%), secretory vesicles (~30%), and plasma membranes (~20%). This pattern suggests that leukolysin overlaps significantly with MMP-9 in the gelatinase granules, but with a dramatically broader distribution than both MMP-8 and MMP-9 by extending to the secretory vesicles

and plasma membrane (~50%). One surprising feature was that nearly 1/5 of leukolysin molecules appeared to be displayed as surface molecules in resting neutrophils (Fig. 10C). To confirm that leukolysin was anchored on neutrophil surface via a putative GPI-anchored protein, intact neutrophils were treated with GPI-PLC. As expected, leukolysin was released into supernatants by GPI-PLC (Fig. 10D, lane 2). Surprisingly, the smaller fragments <40 kDa escaped GPI-PLC mediated release. Nonetheless, these data establish leukolysin as a membrane-associated MMP on neutrophil cell surface.

Discharge of leukolysin by stimulated neutrophils - In response to infections, PMNs are activated to release the contents of their intracellular granules for host defense against invading pathogens (Weiss 1989, Birkedal-Hansen 1993, Borregaard 1997). To implicate leukolysin as part of the released armament, its distribution was monitored in neutrophils treated with PMA for 15 min by subcellular fractionations. As shown Fig. 11, PMA triggered the expected degranulation as indicated by the discharge of markers from azurophil granules, specific granules, gelationase granules and secretory vesicles (Figures 11A to 11G). Specifically, close to 50% of MPO was discharged from azurophil granules (Fig. 11A). Both NGAL and lactoferrin in the specific granules were discharged by more than 80% (Figs. 11B and 11C). Similarly, MMP-9 and albumin were released by PMA stimulation (Figs. 11D and 11E). However, HLA, a marker for plasma membrane, was decreased slightly due to PMN stimulation (Fig.11F). Consistently, almost all leukolysins in specific and gelatinase granules were discharged (Fig. 11 H, lanes α and β1; Figure 11I, lanes 4 vs. 3, 6 vs. 5). As a GPI-anchored protein (Fig. 10) (Kojima 2000), it is expected that the destination of the discharged leukolysins should be cell surface due to the fusion between granule and plasma membranes. Surprisingly, the expected accumulation of leukolysin on the plasma membrane did not materialize because γ band from PMN stimulated neutrophils actually lost ~50% of leukolysin compared to those from resting neutrophils (Fig. 11H, lane γ; Figure 11I, lanes 8 vs. 7). Together, a total of ~75% of cellular leukolysin proteins were discharged in response to PMN treatment. When the supernatants were examined, leukolysin products were detected and estimated to account for

most of the discharged leukolysin from the granules (Fig. 11I, lane 10). These data suggest that a neutrophil actually sheds leukolysin from its membrane rapidly in response to stimulations.

Cytokine and chemokine regulated release of leukolysin from neutrophils - The observed release of soluble leukolysin from PMA-stimulated neutrophils would argue that there must be endogenous regulators capable of mediating similar function under physiological conditions. In fact, a wide range of cytokines and chemokines has been implicated in neutrophil functions by regulating activities such as degranulation, transendothelial and transepithelial migrations (Weiss 1989, Parkos 1997). To further explore the physiological significance of leukolysin expression and release, neutrophils were treated with inflammatory cytokines and chemokines such as IL-1α, β, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -15, -17, -18 and TNFα. Surprisingly, most of these factors appeared to have stimulated the release of leukolysin from neutrophils, albeit with different efficiency. In general two categories of stimulations emerged as represented by cytokines such as IL-1α and β, and chemokine IL-8. As shown in Figure 12, IL-1α and β triggered the release of MMP-9 (Figure 12A, lanes 6, 7), and that of leukolysin with similar efficiency (figure 12B, lane 6, 7). IL-8, the main chemokine for neutrophil chemotaxis in vivo, however, triggered much more robust release of leukolysin and MMP-9 (Fig. 12A and 12B, lanes 8). Interestingly, a new species migrating ~5kDa faster than the 56kDa one was detected only in the IL-8 treated supernatants (Fig. 12B, lane 8, arrow), suggesting that it may be a processed product as observed in MMP-11 (Pei 1994). Thus, cytokines and chemokines may differ not only quantitatively as well as qualitatively the discharge of leukolysin from neutrophils, presumably under different physiological conditions.

Discussion

PMNs are known for their stored arsenal of proteolytic enzymes in host defense and tissue injury (Weiss 1989). It has been assumed for a long time that PMNs only express two secretory MMPs, MMP-8 and MMP-9 (Birkedal-Hansen 1993, Massova 1998). Here evidence is presented indicating that

leukolysin /MT6-MMP /MMP25 is: **a**) a PMN specific enzyme both stored in intracellular granules as well as displayed on cell surface, and **b**) released into extracellular milieu when stimulated with cytokines and chemokines. These features differ dramatically from either MMP-8 or MMP-9. Thus, MMP25 confers on neutrophils a versatile weapon in mediating host defense as well as tissue injury. With the anticipated proteolytic activities against ECM components, leukolysin serves as a target for therapeutical agents against tissue damage in acute inflammations.

Leukolysin as the first MT-MMP for PMNs - Initially, leukolysin is named after the cell populations it is specifically expressed, the human peripheral blood leukocytes, among 26 different tissues and cells screened (Pei 1999c). To begin to uncover its biological function, the cell populations within the leukocyte lineage that express leukolysin was further defined. Surprisingly, only the PMNs, not the lymphocytes nor monocytes, express leukolysin (Fig. 5). This degree of specificity is quite unprecedented given the known complexity in the patterns of expression for MMPs by both normal and diseased cells (Birkedal-Hansen 1993,Nagase 1999, Woessner 1991, Matrisian 1994, Brinckerhoff 1992, Werb 1997). Although leukolysin may not be only expressed by PMNs, the fact that other leukocytes such as monocytes do not express it suggests that it may possess properties that contribute to the functions of PMNs specifically. Interestingly, Velasco and colleagues reported that leukolysin is upregulated in brain tumor samples (Velasco 2000). Since MMPs are known to be dysregulated during carcinogenesis, it is reasonable to reconcile these two observations by suggesting that brain tumor cells or their surrounding stromal cells acquired the ability to express leukolysin during malignant transformations.

Cellular localizations and likely function of leukolysin - Since MMP-8 and MMP-9 are stored in the specific and gelatinase granules of resting PMNs respectively (Murphy 1977 , Hibbs 1985), it was thought that leukolysin would be localized with either or both of them to complement their proteolytic activities. Surprisingly, this assumption is only partly true given the small percentage of leukolysins found in the specific granules, a substantial amount (~40%) in the gelatinase granules, but almost 50% of leukolysins in the secretory

vesicles and plasma membranes (Fig. 9 and 10). In fact, almost 1 out 5 leukolysins appear to be displayed on the cell surface of resting PMNs (Fig. 10). This conclusion was reinforced by the fact that GPI-PLC released leukolysin from PMN cells (Fig. 10). As a GPI-anchored protein, leukolysin joins a class of cell surface molecules expressed by resting PMNs that have been implicated in a wide range of biological functions including signal transduction (Horejsi 1999). On the other hand, the cell surface associated leukolysin may cleave other cell surface molecules such as selectins and growth factors either during migrations or at the inflammatory sites, providing a mechanism of down-regulation after stimulations (Weiss 1989, Werb 1997).

The role of leukolysin in inflammation and tissue damage - The swift discharge of stored leukolysin by chemokine and cytokine treated PMNs (Fig. 12) raises the possibility that leukolysin may be part of the proteolytic enzymes involved in host defense and tissue damages (Weiss 1989, Birkedal-Hansen 1993, Borregaard 1997, Borregaard 1997). Although both cytokines and chemokines are effective in regulating leukolysin secretion, IL-8 is the most effective in triggering such a discharge (Fig. 12), supporting the hypothesis that leukolysin may be important for neutrophil chemotaxis or transmigration through tissues. Although physiological targets for leukolysin remain unknown, leukolysin may help neutrophils negotiate through both the basement membrane and interstition by degrading some of their constituents. Intriguingly, it was shown that leukolysin is an efficient gelatinase (Pei 1999c), and thus may have similar substrate specificity as MMP-2 or 9. Given the report that MMP-9 acts upstream of neutrophil elastase (NE) by proteolytically inactivating NE inhibitor α1PI in experimental bullous pemphigoid (Liu 2000), secreted leukolysin may be able to accomplish the same task as MMP-9. On the other hand, leukolysin may interact with other proteinases in the secreted materials from PMNs, such as neutrophil elastase, PR3 as well as the metalloproteinases, MMP-8 or MMP-9 for zymogen activation (Weiss 1989). In fact, the membrane-type MMPs such as MT1, 3 and 5-MMPs are known activators for the secreted MMPs such as MMP-13 and MMP-2 (for reviews, see Nagase 1999, Massova 1998). Indeed, Velasco and colleagues reported that leukolysin is capable of activating

proMMP-2 in co-transfection experiments (Velasco 2000). Thus, a proteolytic cascade could be formed in the released mixtures of activated PMNs dedicated to the destruction of ECM components at inflammatory sites (Weiss 1989).

Example 3: Implication of leukolysin in asthma effector cells and the engineering of recombinant and active Leukolysin in *E. coli* for high throughput

Leukolysin is found in cells implicated in asthma. For example, leukolysin was expressed in eosinophils and mast cells. RNAs were isolated from neutrophils (Figure 14, lane 1), eosinophils (Figure 14, lane 2), mast cells (Figure 14, lane 3) and macrophage cell line KU-182, reverse transcribed and PCR amplified for leukolysin (Figure 14, upper) and the internal reference gene GAPDH. Note that eosinophils may express the highest amount of leukolysin due to the less amount of RNA used judged from the GAPDH levels (Figure 14, upper and lower part of lane 2). Surprisingly, mast cells are also sources of leukolysin (Figure 14, lane 3)

Also, recombinant leukolysin in *E. coli* was expressed and regenerated. The catalytic domain (aa107-280) was cloned into a modified pET15b vector (pET15bSma1) and the resultant plasmid was transformed into DE3 cells for expression. The cells were lysed by sonication and leukolysin protein was purified in the inclusion body. In Figure 15, from lane 1 to 5, the purified inclusion body from the induced DE3:pET15b Sma1 -Leukolysin were sequentially diluted in TC buffer (50mM Tris pH7.5, 5mM CaCl2, 10uM ZnCl, 1% Triton X-100). Lane 6 was a direct dilution of the purified protein in TC buffer in a 1:81 ration. Lanes 7, 8, 9 are similar dilution as in lane 6, but at different time. Note that the purified materials can be refolded to generate proteolytic activity (white band). The arrow indicates the purified leukolysin. The arrow head indicates the main proteolytic species from the purified one.

It was also shown that refolded leukolysin can cleave substrates in solution. While zymography in Fig. 15 can detect any proteolytic species whether they are latent or active, it was necessary to ascertain that the refolded leukolysin is active. To accomplish this, a solution assay was adopted to show

that three different preparations of leukolysin (Figure 16, lanes 3-5) could all degrade the substrate (gelatin, Figure 16, lane 1) efficiently in a 12 h incubation period. Further more, MMP inhibitors such as synthetic BB94 or natural TIMP1 and 2 (Figure 16, lanes 7-10) could completely block its activity.

All publications, patents, and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

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What is claimed is:

1. A polypeptide comprising matrix metalloproteinase 25 (MMP25, also called MT6-MMP or leukolysin).

2. The polypeptide of claim 1 covalently linked to a carrier molecule.

3. A polynucleotide comprising a sequence encoding, or complementary to, MMP25.

4. An expression vector comprising a polynucleotide encoding, or complementary to, MMP25.

5. A transgenic cell containing an expression vector comprising a polynucleotide encoding, or complementary to, MMP25.

6. A method for treating a leukolysin-associated condition comprising administering to an afflicted human an amount of a therapeutic preparation comprising a compound effective to counteract the symptoms of the condition.

7. The method of claim 6, wherein the animal is a human.

8. The method of claim 6, wherein the leukolysin-associated condition is 1) acute or chronic inflammation caused by neutrophils; 2) autoimmune diseases where many neutrophil proteins are autoantigens; 3) asthma other related immune disorders caused by granulocytes; 4) tissue injury and organ failure mediated by MMP25 expressing cells such as granulocytes; 5) malignant cancers where MMP25 enables tumor cells to invade and metastasize through its ability to degrade extracellular matrix; 6) genetic disorders with defects in neutrophil deficiency; 7) organ transplant patients with episodes of granulocyte infiltrations; 8) organ

failure due to attacks by neutrophils or neutrophil-related immune responses; 9) cardiovascular conditions or damages mediated by neutrophils or its discharged contents; 10) lung damage mediated by neutrophils; 11) skin irritations and blister where granulocytes mediate the destruction of connected tissues; 12) allergy associated with eosinophils and mast cells; 13) arthritis; 14) chronic bronchitis; 15) pulmonary emphysema; 16) α-1 anti-trypsin deficiency; 17) cystic fibrosis; 18) idiopathic pulmonary fibrosis; 19) adult respiratory distress syndrome; 21) familial Mediterranean fever; or 22) periodontitis and oral ulcers.

9. The method of claim 8, wherein the organ failure is renal failure.

10. The method of claim 8, wherein the neutrophil-associated pulmonary disease is chronic obstructive pulmonary disease.

11. The method of claim 6, wherein the compound is TIMP-1, TIMP-2, TIMP-3, TIMP-4 or their closely related inhibitors; a BB-94 inhibitor; catalytically inactive leukolysin; hemopexin-like domain of leukolysin; or natural herb compounds that bind zinc, thereby removing zinc from leukolysin.

12. The method of claim 6 wherein the therapeutic preparation is administered in combination with a pharmaceutically acceptable liquid vehicle.

13. The method of claim 6 wherein the therapeutic preparation is administered by spraying or by nebulization.

14. A method for stimulating the activation of neutrophils in an animal in need thereof, comprising administering to an animal a therapeutic amount of a composition comprising polypeptide comprising a sequence

encoding MMP25 to the animal effective to stimulate the activation of neutrophils in the animal.

15. The method of claim 14, wherein the compound is a cytokine or chemokine; a modified version of leukolysin exhibiting heightened activities; G-CSF or GM-CSF, which can upregulate leukolysin expression; or transcriptional factor in the class of C/EBPs, which controls the transcription of myeloid lineage.

16. The method of claim 14, wherein the compound is IL1, 2, 3, 4, 6, 7, 8, 9, 10, 11, or 12.

17. A method for mediating the destruction of a pathogen or infected host tissues in an animal in need thereof, comprising administering to an animal a therapeutic amount of a composition comprising polypeptide comprising a sequence encoding MMP25 to the animal effective to mediate the destruction of the invading pathogens or the infected host tissues in the animal.

18. The method of claim 17, wherein the pathogen is a virus, bacterium, fungus or parasite.

19. A method for inhibiting the activation of neutrophils in an animal in need thereof, comprising administering to an animal a therapeutic amount of a composition comprising polypeptide comprising a sequence encoding MMP25 to the animal effective to inhibit the activation of neutrophils in the animal.

20. The method of claim 19, wherein the animal has an inflammatory disease.

21. The method of claim 20, wherein the inflammatory disease is a fever, periodontal disease, cystic fibrosis, familial Mediterranean fever, arthritis, or emphysema.

22. A method for inhibiting an over-reactive immune system response in an animal in need thereof, comprising administering to an animal a therapeutic amount of a composition comprising polypeptide comprising a sequence encoding MMP25 to the animal effective to inhibit the over-reactive immune system response in the animal.

23. The method of claim 22, wherein the over-reactive immune system response is caused by allergy or an autoimmune disease.

24. The method of claim 23, wherein the autoimmune disease is arthritis.

25. A method of suppressing tumor invasion and metastasis comprising administering to individuals with malignancy a composition comprising natural or synthetic inhibitors of MMP25, or an antibody specific for MMP25, or a poly- or oligo-nucleotide that can block the activity of MMP25, in order to attenuate the mobility of cells expressing MMP25.

26. A method of stimulating neutrophils comprising administering to an animal experiencing heavy infections composition comprising MMP25 to the animal effective to mediate the destruction of invading pathogens.

27. An antibody that specifically binds isolated, purified MMP25, or a portion thereof.

28. The antibody of claim 27, wherein the antibody is a monoclonal or polyclonal antibody.

29. The antibody or fragment thereof of claim 27, wherein the fragment thereof is an Fab, $F(ab')_2$, or Fv fragment.

30. The antibody or fragment thereof according to claim 27, wherein the antibody or fragment thereof is attached to a substrate.

31. The antibody or fragment thereof according to claim 30, wherein the substrate is a gel, hydrogel, resin, bead, nitrocellulose, nylon filter, microtiter plate, culture flask, or polymeric material.

32. The antibody or fragment thereof of claim 27, further comprising a detectable moiety.

33. The antibody or fragment thereof of claim 32, wherein the detectable moiety is a radionuclide, enzyme, specific binding pair component, colloidal dye substance, fluorochrome, chemiluminescent substance, electrochemiluminescent substance, electroactive agent, reducing substance, latex, digoxigenin, metal, particulate, dansyl lysine, antibody, protein A, protein G, electron dense material, or chromophore.

34. A pharmaceutical composition comprising an amount of an antibody, or a fragment or a mixture thereof, that specifically binds to MMP25, wherein the amount is effective to inhibit a neutrophil response in a recipient with acute or chronic inflammation and tissue injury.

35. A continuous cell line that produces an antibody that specifically recognizes a target peptide, wherein the target peptide is leukolysin.

36. The continuous cell line of claim 35, wherein the cell line is a monoclonal antibody cell line.

37. The continuous cell line of claim 35, wherein the target peptide is covalently linked to a carrier molecule.

38. The continuous cell line of claim 37, wherein the carrier molecule is keyhole limpet hemocyanin (KLH).

39. An animal that produces polyclonal antibodies that specifically recognizes a target peptide, wherein the target peptide is leukolysin.

40. The animal of claim 39, wherein the target peptide is covalently linked to a carrier molecule.

41. A diagnostic method for detecting a leukolysin-associated condition in a patient comprising:

(a) contacting a physiological sample suspected of containing leukolysin to form a purified leukolysin sample with an amount of detection agent specific for leukolysin to form an leukolysin:detection agent complex; wherein the detection agent is an antibody or fragment thereof that specifically recognizes leukolysin; and

(b) detecting the presence or amount of leukolysin:detection agent complex present in the sample to determine whether the patient has inflammatory disease.

42. The diagnostic method of claim 41, wherein the physiological sample is plasma, serum, tears, urine, whole gut lavage, lung lavage, peritoneal lavage, skin blister fluids, mucus, feces, GI tract tissue including mucosa and submucosa, or jejunal effluent.

43. The diagnostic method of claim 41, wherein the leukolysin-associated condition is 1) acute or chronic inflammation caused by neutrophils; 2) autoimmune diseases where many neutrophil proteins are autoantigens; 3) asthma other related immune disorders caused by granulocytes; 4)

tissue injury and organ failure mediated by MMP25 expressing cells such as granulocytes; 5) malignant cancers where MMP25 enables tumor cells to invade and metastasize through its ability to degrade extracellular matrix; 6) genetic disorders with defects in neutrophil deficiency; 7) organ transplant patients with episodes of granulocyte infiltrations; 8) organ failure due to attacks by neutrophils or neutrophil-related immune responses; 9) cardiovascular conditions or damages mediated by neutrophils or its discharged contents; 10) lung damage mediated by neutrophils; 11) skin irritations and blister where granulocytes mediate the destruction of connected tissues; 12) allergy associated with eosinophils and mast cells; 13) arthritis; 14) chronic bronchitis; 15) pulmonary emphysema; 16) α-1 anti-trypsin deficiency; 17) cystic fibrosis; 18) idiopathic pulmonary fibrosis; 19) adult respiratory distress syndrome; 21) familial Mediterranean fever; or 22) periodontitis and oral ulcers.

44. The method of claim 41 wherein the peptide is covalently linked to a carrier molecule.

45. The method of claim 44, wherein the carrier molecule is keyhole limpet hemocyanin (KLH).

46. The method of claim 41 wherein the antibody or fragment thereof is an Fab, $F(ab')_2$, or Fv fragment.

47. The method of claim 41, wherein the antibody or fragment thereof is attached to a substrate.

48. The method of claim 41, wherein the substrate is a gel, hydrogel, resin, bead, nitrocellulose, nylon filter, microtiter plate, culture flask, or polymeric material.

49. The method of claim 41, wherein the antibody or fragment thereof further comprises a detectable moiety.

50. The method of claim 49, wherein the detectable moiety is a radionuclide, enzyme, specific binding pair component, colloidal dye substance, fluorochrome, reducing substance, chemiluminescent substance, electrochemiluminescent substance, electroactive substance, latex, digoxigenin, metal, particulate, dansyl lysine, antibody, protein A, protein G, electron dense material, or chromophore.

51. The method of claim 41, wherein the detection step (b) is by enzyme-linked immunosorbent assay, immunonephelometry, agglutination, precipitation, immunodiffusion, immunoelectrophoresis, immunofluorescence, electrochemiluminescence, surface plasmon resonance, chemiluminescence, electrochemical immunoassay, radioimmunoassay, or immunohistochemistry.

52. A method for monitoring the treatment of leukolysin-related disease in a patient comprising:

(a) contacting a physiological sample suspected of containing leukolysin with an amount of detection agent specific for leukolysin to form an leukolysin:detection agent complex

(b) detecting the amount of leukolysin:detection agent complex present in the sample;

(c) repeating steps (a) and (b) at a point later in time; and

(d) comparing the amounts determined in steps (b) and (c), and correlating the change in the amounts to determine whether inflammation is diminishing.

53. A kit for the detection of inflammatory disease in a patient, the kit comprising:

(a) a composition comprising a detection agent specific for leukolysin, wherein the detection agent is an antibody or fragment thereof that specifically recognizes the leukolysin peptide; and

(b) packaging materials enclosing the composition.

Figure 1A

A

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MRLRLRLLALLLLLLAPPARAPKPSAQDVSLGVDWLTRYGYLPPPHPAQAQLQSPEKLRD  60
AIKVMQRFAGLPETGRMDPGTVATMRKPRCSLPDVLGVAGLVRRRRRYALSGSVWKKRTL 120
TWRVRSFPQSSQLSQETVRVLMSYALMAWGMESGLTFHEVDSPQGQEPDILIDFARAFHQ 180
DSYPFDGLGGTLAHAFFPGEHPISGDTHFDDEETWTFGSKDGEGTDLFAVAVHEFGHALG 240
LGHSSAPNSIMRPFYQGPVGDPDKYRLSQDDRDGLQQLYGKAPQTPYDKPTRKPLAPPPQ 300
PPASPTHSPSFPIPDRCEGNFDAIANIRGETFFFKGPWFWRLQPSGQLVSPRPARLHRFW 360
EGLPAQVRVVQAAYARHRDGRILLFSGPQFWVFQDRQLEGGARPLTELGLPPGEEVDAVF 420
SWPQNGKTYLVRGRQYWRYDEAAARPDPGYPRDLSLWEGAPPSPDDVTVSNAGDTYFFKG 480
AHYWRFPKNSIKTEPDAPQPMGPNWLDCPAPSSGPRAPRPPKATPVSETCDCQCELNQAA 540
GRWPAPIPLLLLPLLVGGVASR                                        562
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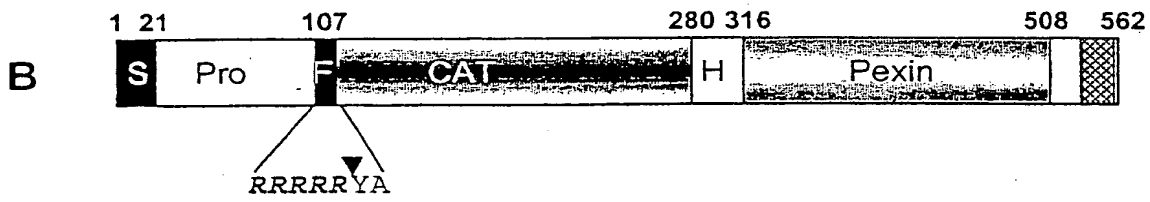


Figure 1B

Figure 1C

C

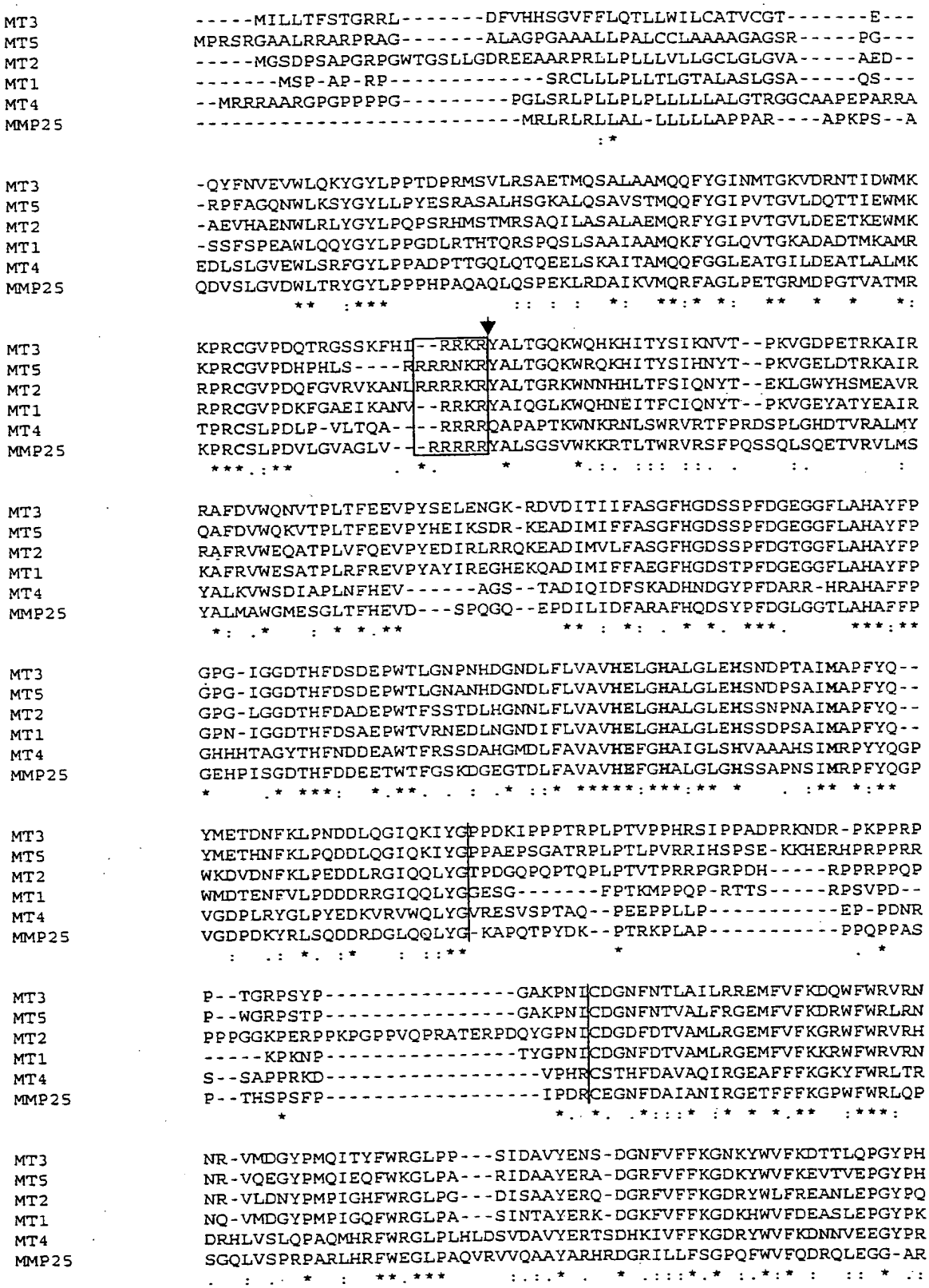
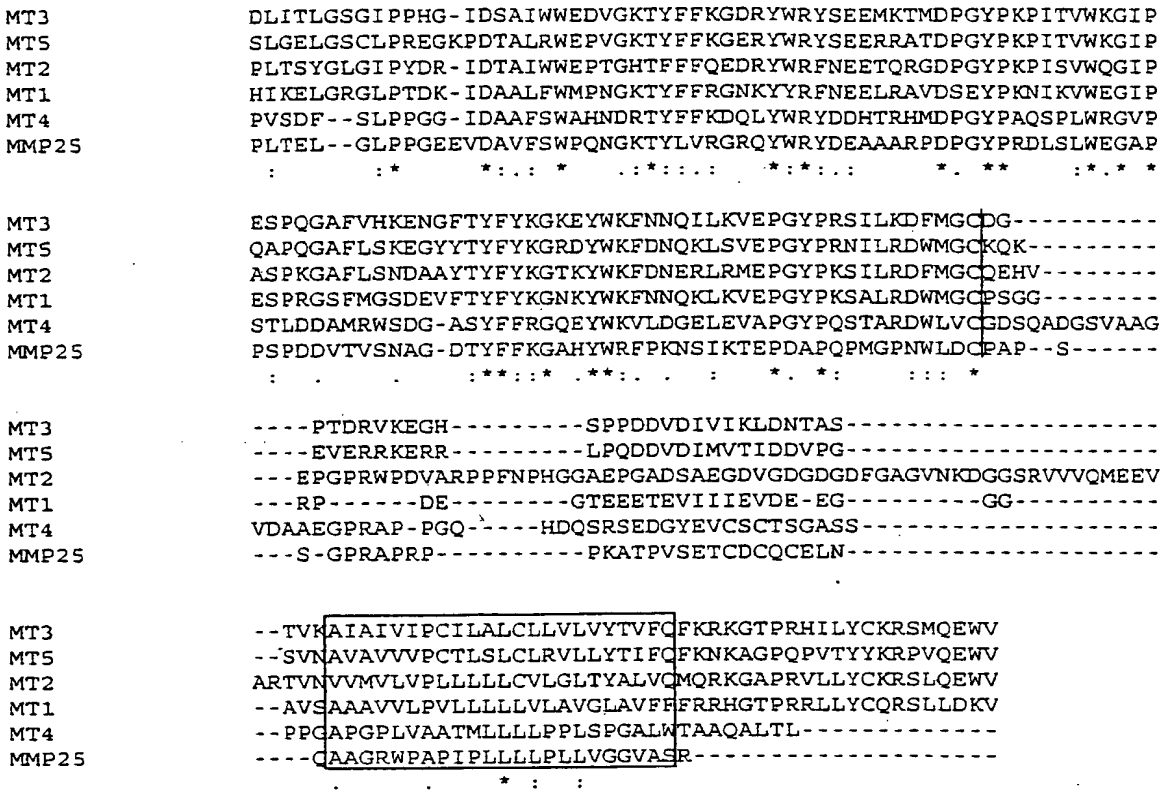


Figure 1C cont.



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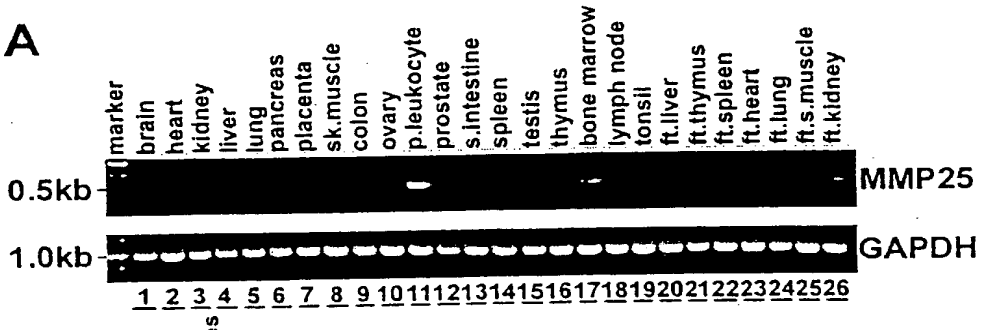

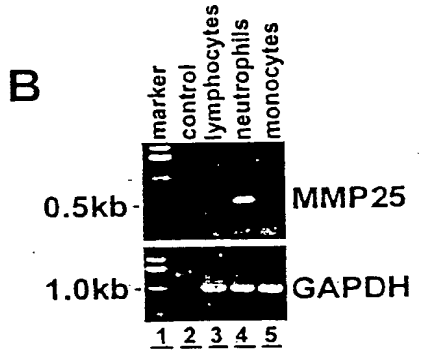

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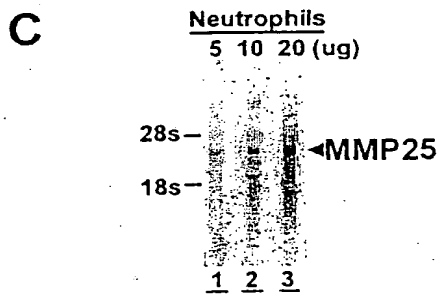

Figure 2C

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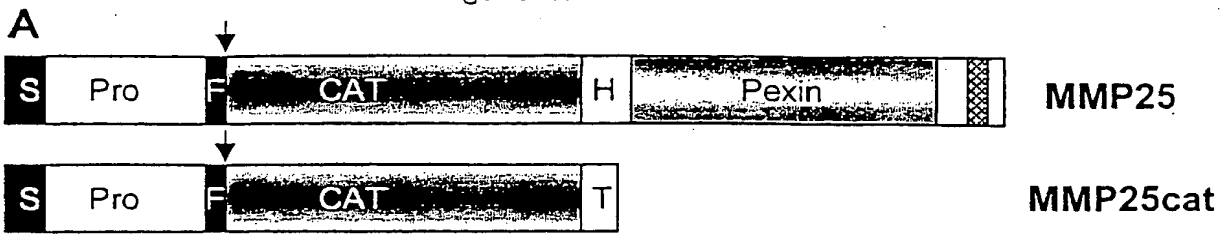

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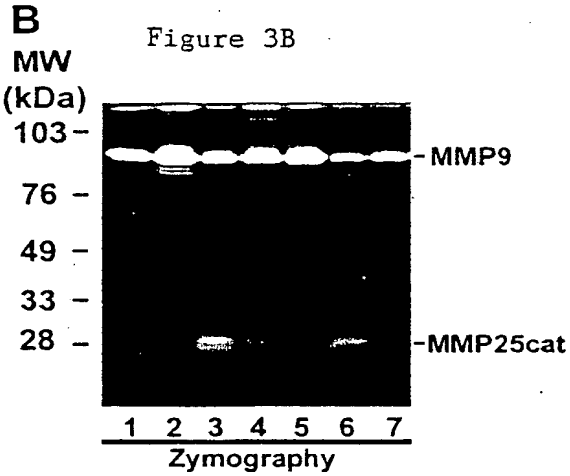

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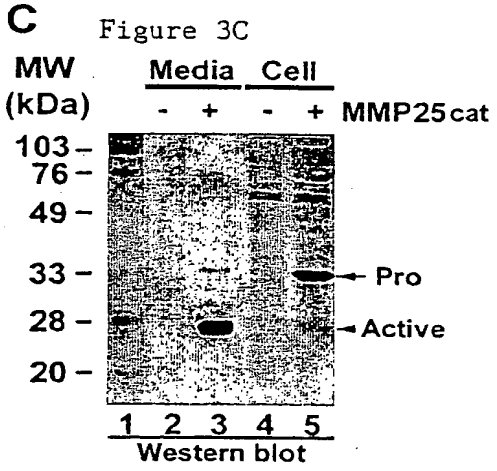

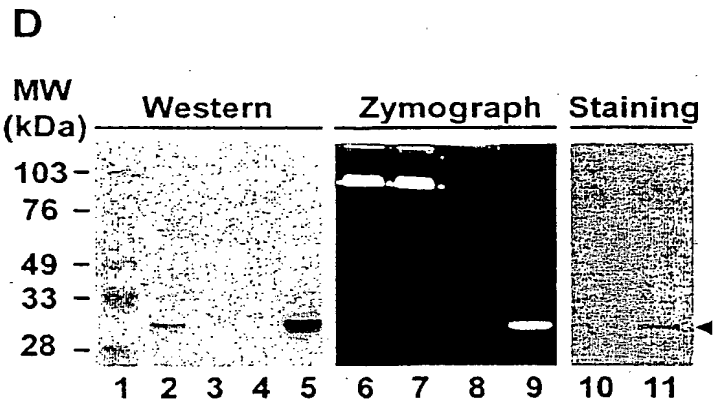

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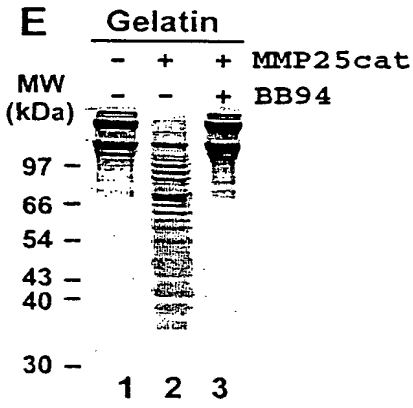

Figure 3E

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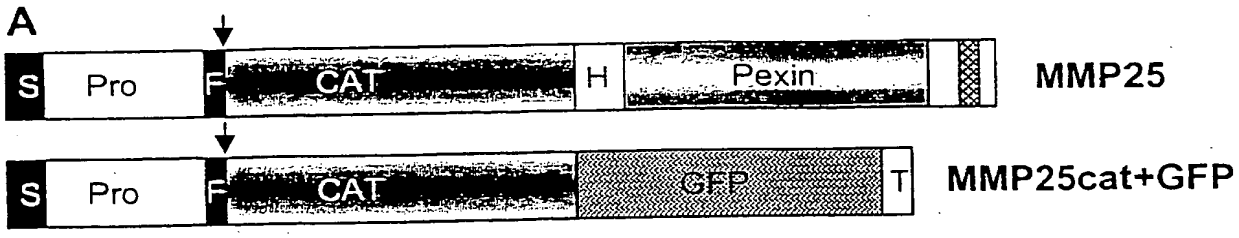

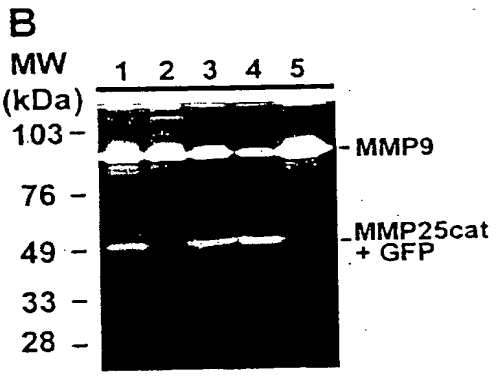

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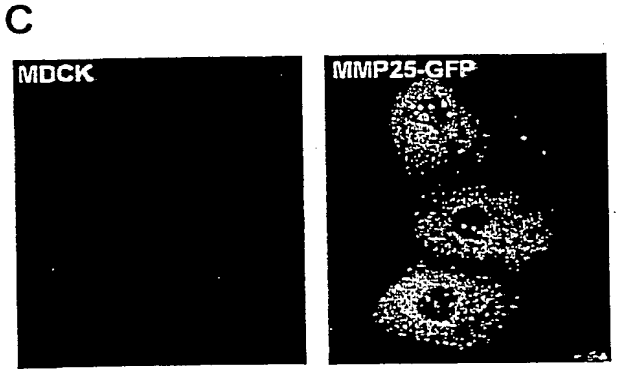

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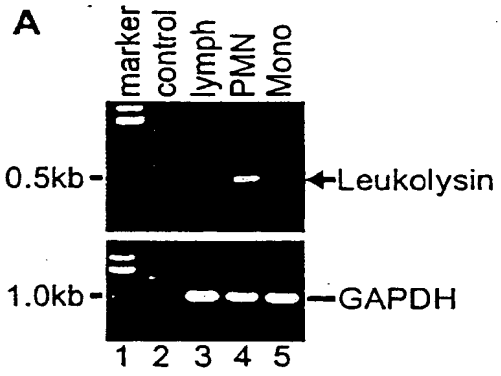

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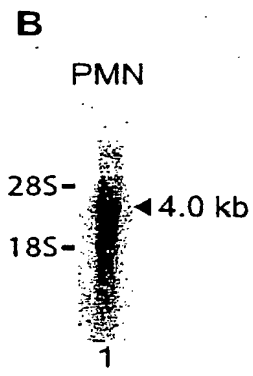

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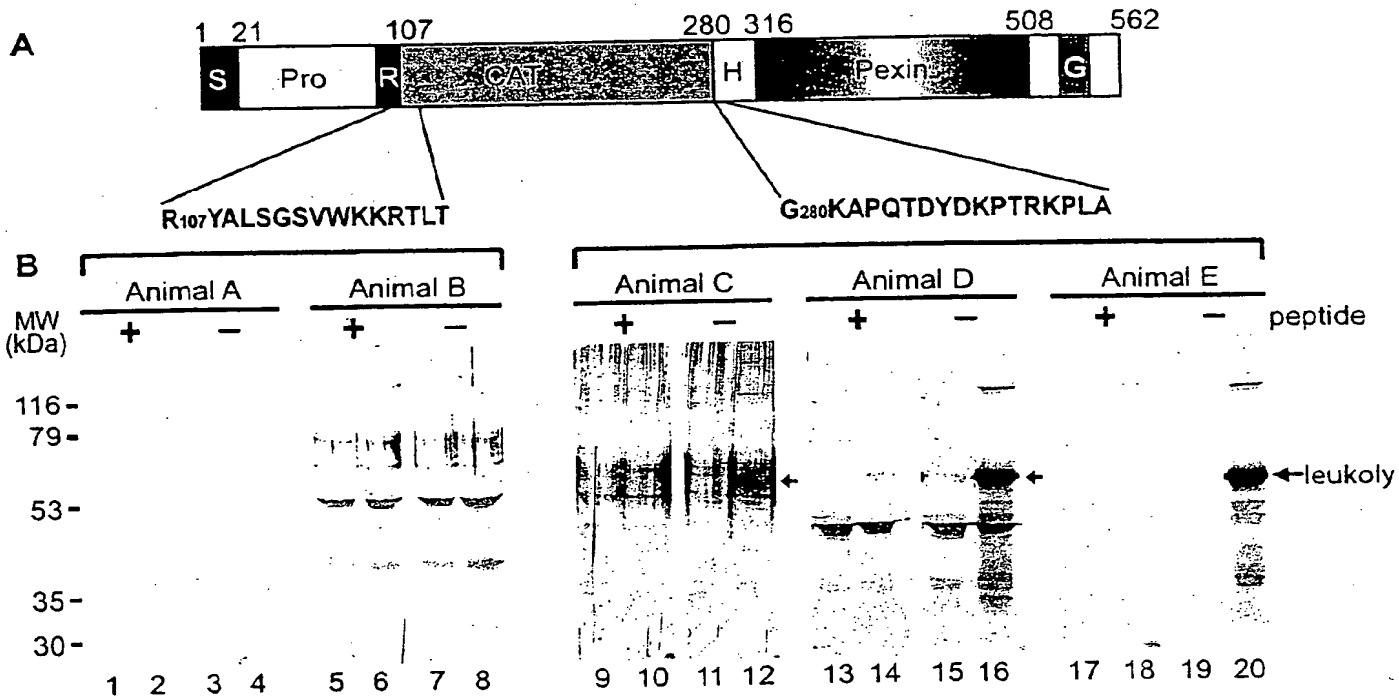

Figure 6B

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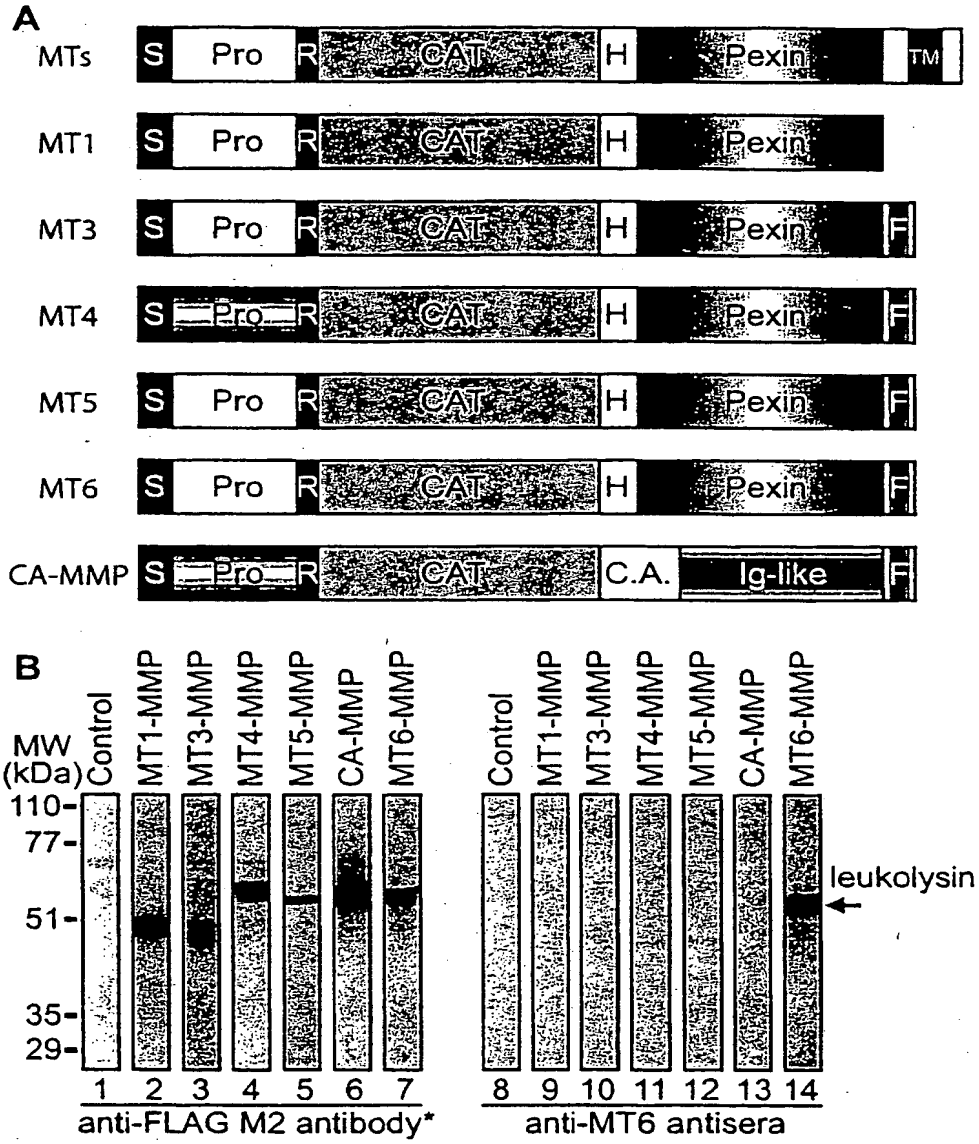
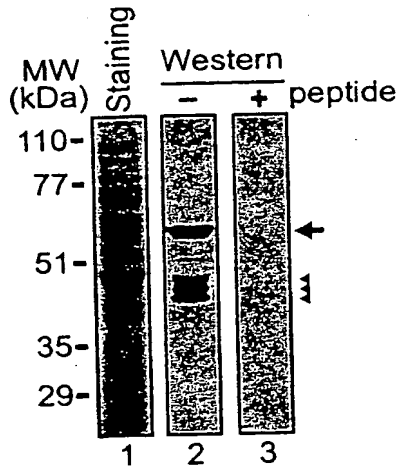


Figure 7B

Figure 8



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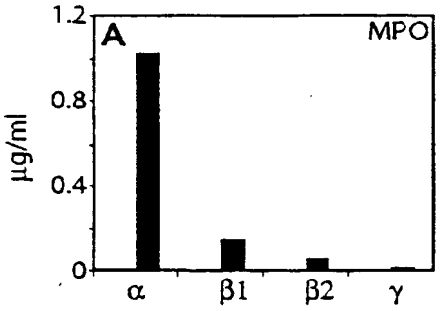


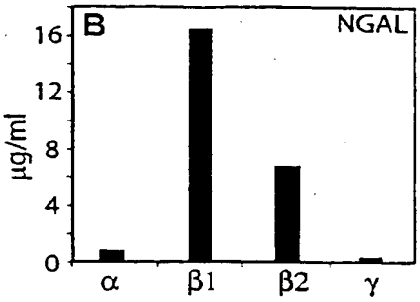
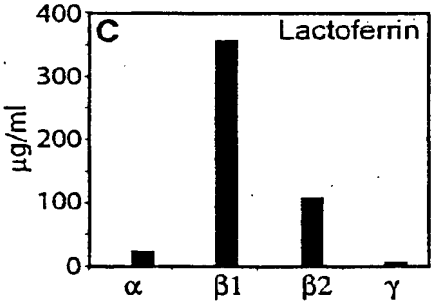


Figure 9B

Figure 9C



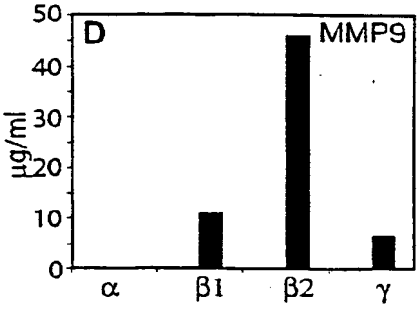
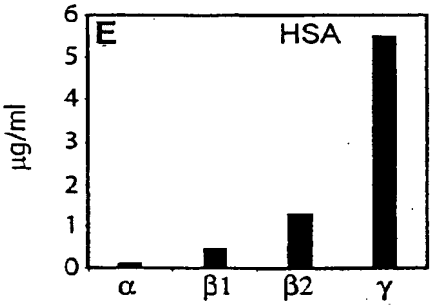


Figure 9D

Figure 9E



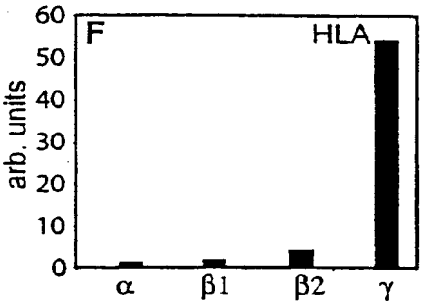
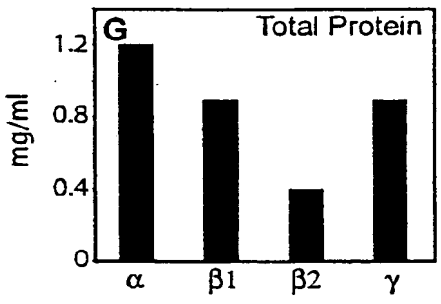


Figure 9F

Figure 9G



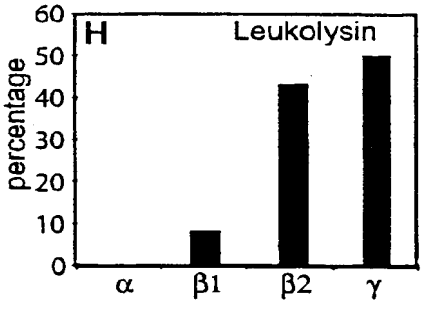


Figure 9H

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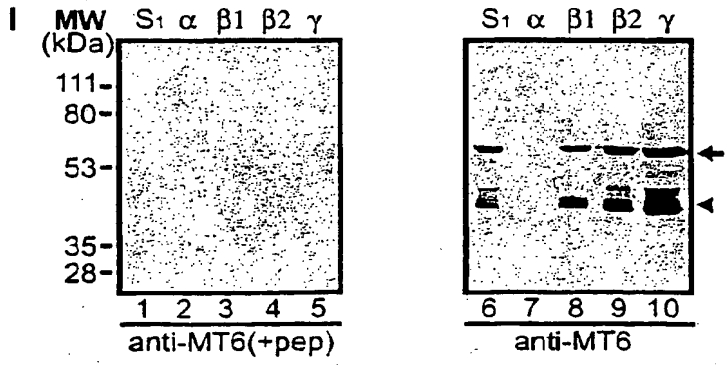

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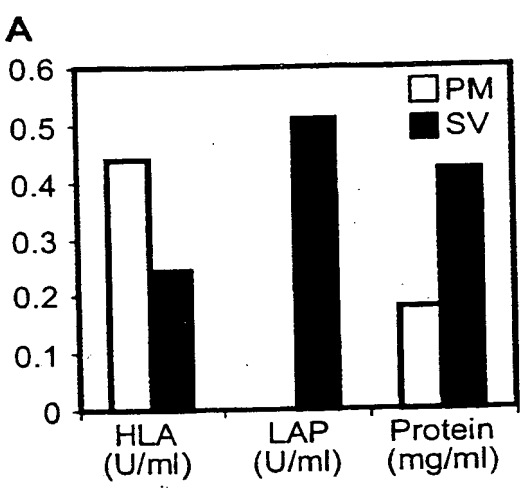

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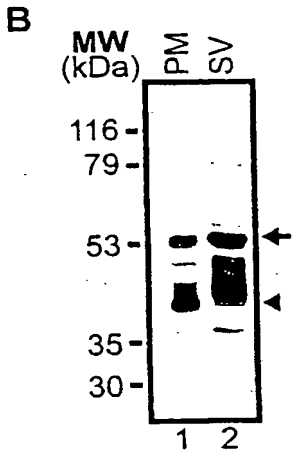

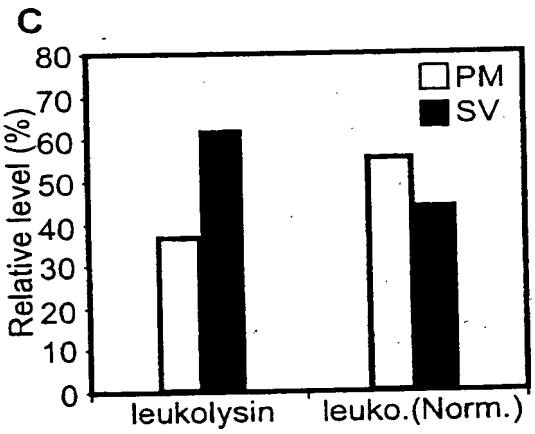

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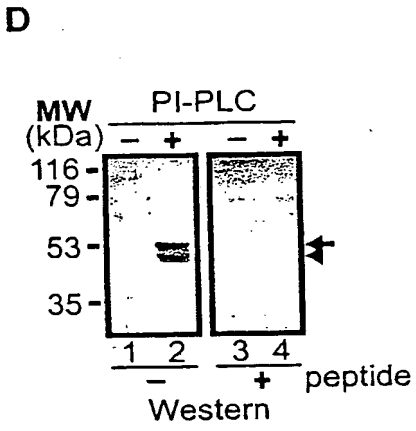

Figure 10D

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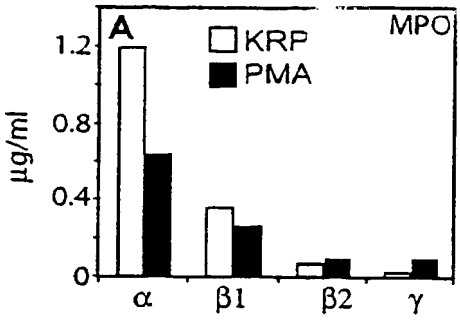

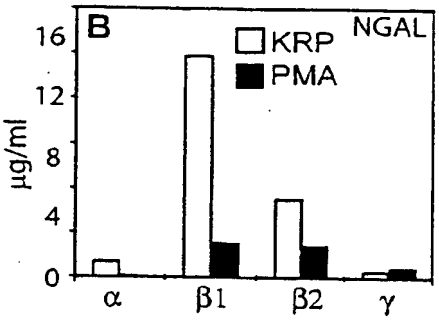

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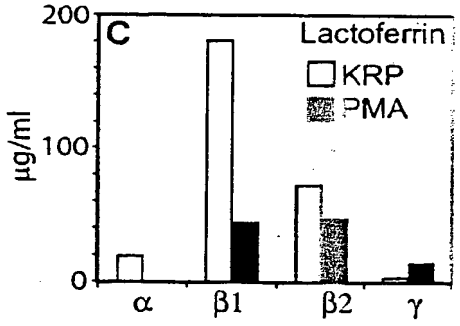

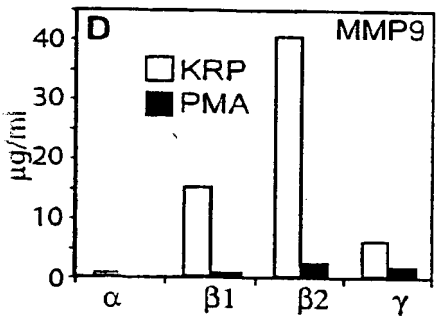

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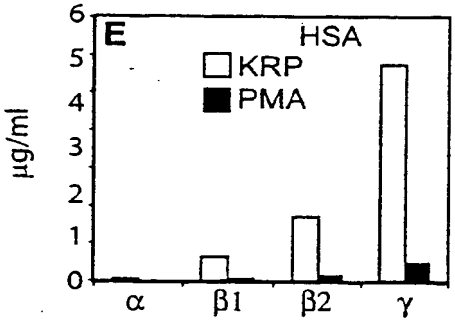

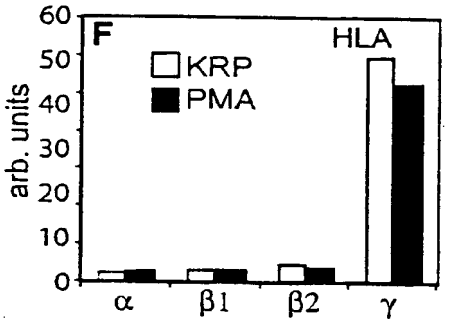

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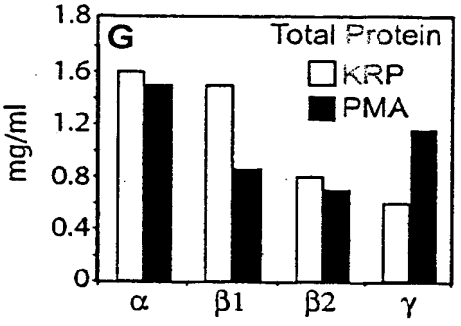

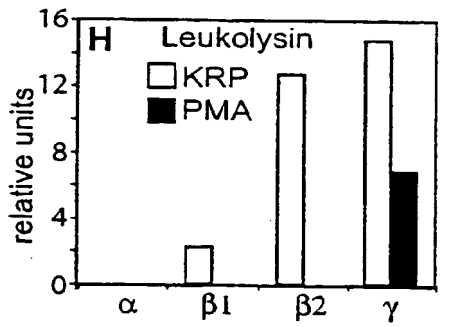

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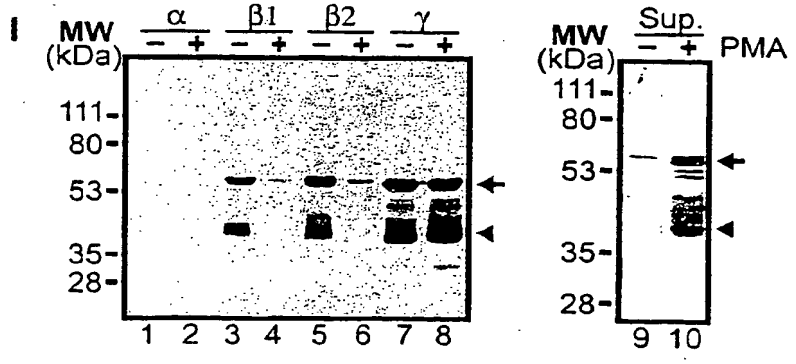
Figure 11I



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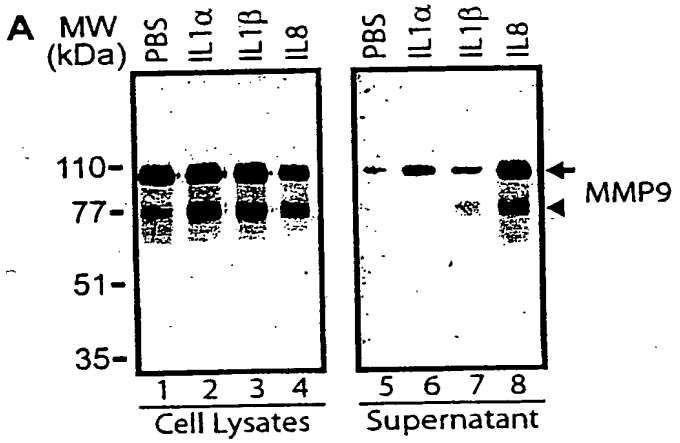

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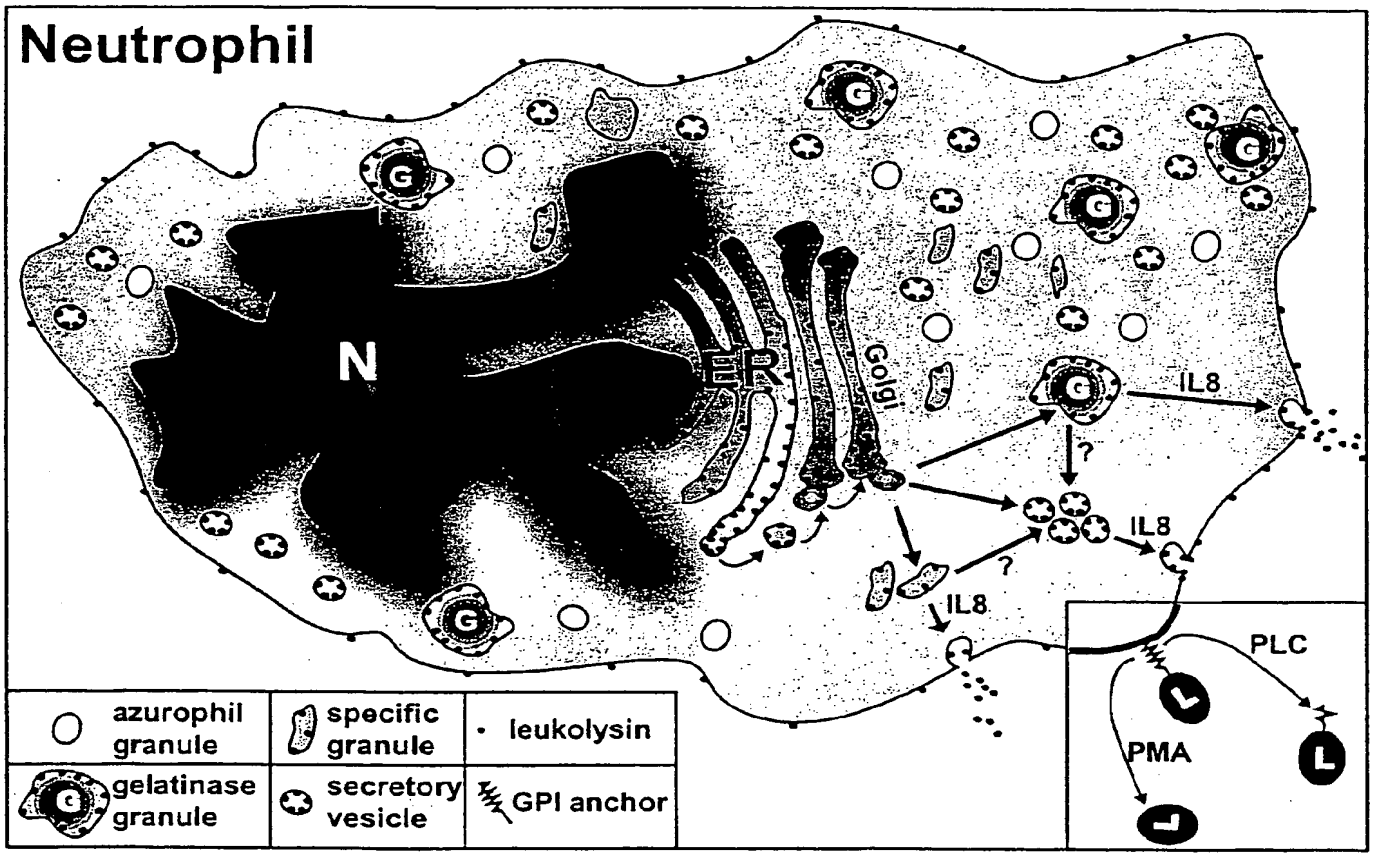

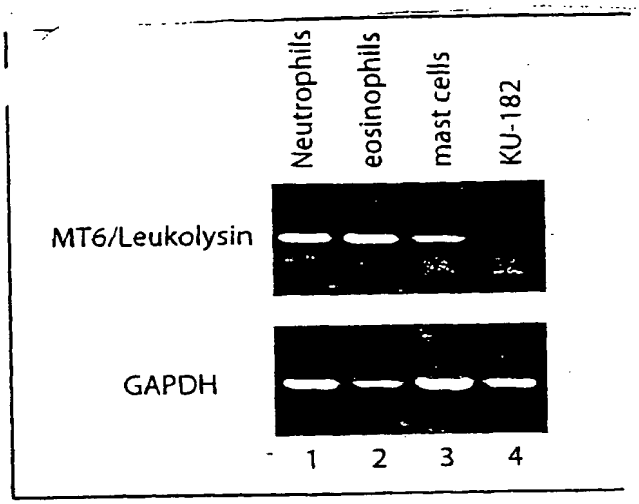

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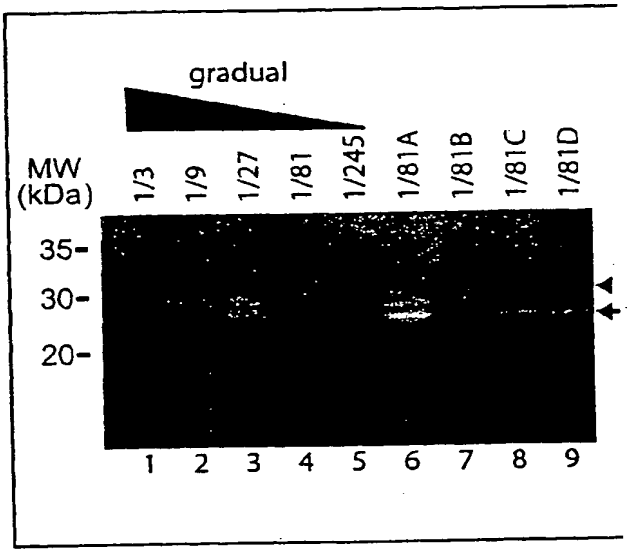

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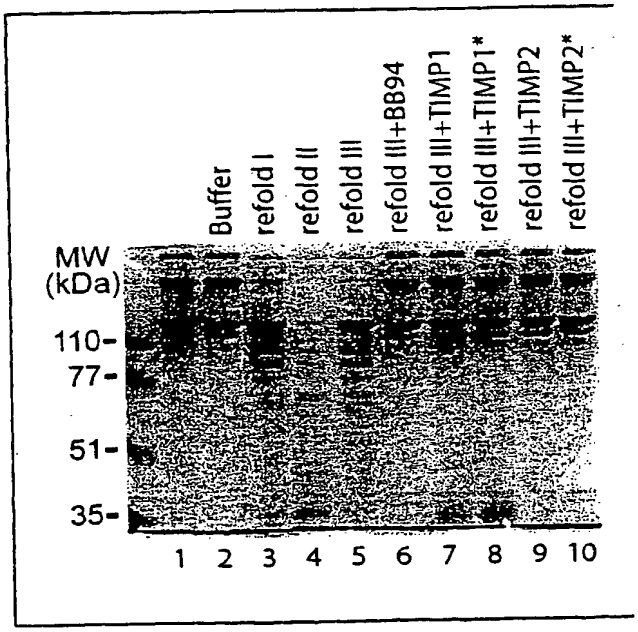

Figure 16

SEQUENCE LISTING

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Pei, Duanqing

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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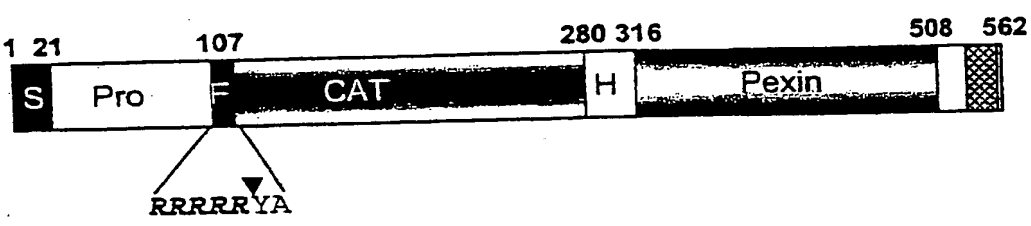
(54) Title: LEUKOLYSIN/MMP25/MT6-MMP



(57) Abstract: A novel compound, leukolysin, and therapeutic methods for treating conditions associated with the presence or absence of leukolysin is provided. Also provided are methods to detect or monitor inflammatory disease by determining the presence or amount of leukolysin in a physiological sample.

WO 01/42438 A3

INTERNATIONAL SEARCH REPORT

In. .ational Application No
PCT/US 00/33763

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/57 C12N9/64 A61K38/48 C07K16/40 C07K17/00
G01N33/532 G01N33/53 C12N5/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 35276 A (SCHERING CORP) 15 July 1999 (1999-07-15) SEQ ID No:4 the whole document ---	1-7, 11-13, 27-40, 52,53
X	PEI D: "Leukolysin/MMP25/MT6-MMP:a novel matrix metalloproteinase specifically expressed in the leukocyte lineage" CELL RESEARCH, vol. 9, no. 4, December 1999 (1999-12), pages 291-303, XP001000899 cited in the application the whole document --- -/--	1-53

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2001	27/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bilang, J

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	DATABASE EMBL 'Online! Accession Number AI150017, 1 October 1998 (1998-10-01) "Soares testis NHT Homo sapiens cDNA clone" XP002169331 cited in the application abstract	1-5, 27-33, 35-40
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INTERNATIONAL SEARCH REPORT

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